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DEHYDROGENASE ACTIVITY AND RESPIRATION; A QUANTITATIVE COMPARISON[1,2]

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The evidence that the tricarboxylic acid cycle of Krebs plays an important part in plant metabolism is now very extensive (see the review of Burris (8)). It appears that all the necessary enzymes are present, at least in those few tissues which have been examined (12, 20, 21, 23, 25), and the oxidation of organic acids by in vitro preparations appears to proceed by way of the cycle (7, 12). However, the evidence as to whether the cycle functions as the main route of respiration in vivo is less convincing. In yeast, Krebs, Gurin and Eggleston (18) have shown that the cycle "cannot be the main mechanism of oxidation," but is rather a path for the synthesis of intermediary metabolites. Roberts et al (26) have recently come to a similar conclusion for *Escherichia coli*.

As to the in vivo role of the cycle in plants the evidence is still more incomplete, though there are several observations which are hard to reconcile with any central role for the cycle in the respiratory system. One of these is the relative insensitivity of respiration to malonate, found, for example, in freshly harvested oat coleoptiles (1, 4) and particularly in carrot roots (15). It is still more marked in Euglena; for Eichenberger, in unpublished experiments in this laboratory, has found that malonate does not inhibit the endogenous respiration even at pH 3.5, where it would be largely in the undissociated malonic acid form. Nevertheless, the oxidation of added succinate was powerfully inhibited by malonate under exactly the same conditions. Another difficulty is the failure of labeled organic acids to come into equilibrium with all of the cycle intermediates in tobacco leaves (29).

It seems therefore desirable to obtain further information as to the quantitative importance of the cycle in plant respiration.

If the activity of an enzyme in vitro is in excess of the rate of respiration of the tissue from which it was extracted, it may be concluded (with reservations) that this enzyme is non-limiting in respiration. The main reservation is that the conditions for the activity of the enzyme in vivo are no less favorable than those in vitro. Considering the degree of organization of the cell contents, this reservation does not seem to be an overriding objection to the formation of positive conclusions. However, if it is found that the enzyme activity in vitro is relatively *less* than the rate of respiration, the obverse conclusion would be open to serious doubts.

In comparing enzyme activities with rates of respiration, there arises immediately the problem of matching the conditions in the cytoplasm. With present knowledge it is obviously impossible to evaluate with precision even a single variable such as cytoplasmic (or mitochondrial) pH. However, a reasonable starting point is to compare respiration rate with the *optimal* rate of action of the extracted enzyme, invoking known or reasonable factors when available. For instance, the concentrations of coenzyme I and ATP where required in the experiments reported below are adjusted to 10^{-3} M. These may seem high, but the extraordinary activity of cofactors in intact mitochondria (16) and the upward revision of the estimates of coenzyme I and II concentrations in plants (2) lend support to this procedure. The role of other factors in establishing optimal rates was discussed in the previous paper (25).

While a comparison between respiration and the activities of individual enzymes of the cycle cannot be truly quantitative, moderately accurate assays together with the determination of optimal conditions for the enzymes involved are clearly needed. At least in etiolated tissues, these are available for alpha-ketoglutaric, succinic, and malic dehydrogenases (25).

As a basis for comparison, a measure is needed of the maximum level of respiration of the intact tissue (at the same temperature). Fortunately there have been many studies of the optimal conditions for plant respiration, so that the maximum rate of respiration of a tissue can be determined with fair certainty.

Having arrived at a figure for the rate of respiration, the next question is: how great must the activity of the cycle enzymes be in order to allow for their mediating the entire respiration? Since the cycle is composed of five dehydrogenations, it follows that in a respiratory system proceeding by it exclusively, each dehydrogenation would account for one fifth of the total oxygen uptake. However, one "turn" of the cycle results in the oxidation of one molecule of pyruvate,

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with an RQ of 1.2, while the tissues here studied have a lower overall RQ; values as low as 0.8 were reported for the oat coleoptile (1), while pea stem internodes in auxin average 0.84 (10). Evidently, therefore, at least one other dehydrogenation outside the cycle must contribute to the respiration, and presumably this comprises the step between triose and pyruvate. Hence each dehydrogenation in the cycle only needs to proceed at one sixth of the total rate of oxygen consumption. In the comparisons between dehydrogenase activity and respiration which follow, the theoretical rate of substrate oxidation will therefore be considered as one sixth the oxygen uptake of the intact tissue.

Methods and Materials

The enzyme assays, employing both the Thunberg and the manometric techniques, were described in detail in a preceding communication (25). Unless otherwise stated, conditions were as given therein. The substrate concentrations were 0.02 M throughout. For malic dehydrogenase (Thunberg) DPN was normally 10^{-3} M. For malic and alpha-ketoglutaric "oxidases" DPN and ATP were both 10^{-3} M. For the Thunberg tests the redox dye was 10^{-4} M 2,6-dichlorophenolindophenol; equivalent oxygen uptake is calculated from the rate of dye reduction. The addition of cytochrome c under the conditions employed was not required.

The tissues chosen for study were leafless coleoptiles of 3-day-old oat seedlings (*Avena sativa* var. "Segerhavre") and the third internodes of 7-day-old etiolated pea seedlings (*Pisum sativum* var. "Alaska"). The conditions of growth were those which have been standardized for auxin assays, and are described in detail elsewhere (6, 9).

The optimal conditions for respiration of coleoptiles as described by J. Bonner (5) are: 0.01 M KH_2PO_4 (pH 4.5), 2% sucrose, 100 mg/l $MnSO_4$, 0.005 M pyruvate, and 3 mg/l indoleacetic acid. Pea stem internodes appear to be adequately provided with endogenous sugars, and are therefore relatively insensitive to added nutrients or cofactors other than auxin; nevertheless, identical solutions were employed, except that indoleacetic acid was 1 mg/l.

Respiration was measured with samples of up to 250 mg fresh weight (FW) of diced tissue. Under the conditions employed, the rates were not limited by oxygen tension. Oxygen uptake was linear for at least 1 hour. All measurements of both respiration and dehydrogenase activity were conducted at 25° C.

The following abbreviations are employed: FW, fresh weight of original tissue; M_w, washed mitochondrial fraction; Q_{O_2} (N), microliters O_2 exchanged per hour per mg N; Q_{O_2} (FW), microliters O_2 exchanged per hour per gram FW; DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate.

Experimental

Efficiency of Extraction: The activity of separate respiratory enzymes can only be truly compared with the rate of respiration if three requirements are fulfilled: the extraction of the enzyme must be quantitative, the measurement of activity of the enzymes must be quantitative, and the level of respiration must be maximal.

The third of these requirements has been aproximated by employing conditions which yield the highest rate of oxygen uptake. The rates obtained compare favorably with those in other published reports. The first and second requirements were considered in a previous paper (25) and are explored here in further detail.

The chilled and diced tissue was ground in a mortar at 3° C with 2 to 5 volumes of sucrose and phosphate solution, and extracted three times further with smaller amounts of solution. It was reported in the previous paper, and has been confirmed since, that the residue from coleoptiles prepared in this manner is essentially free from succinic dehydrogenase activity. Pea stem sections, however, contain a higher ratio of vascular tissue to parenchyma, and therefore were not

Table I

Residual Activity after Extraction of Pea Stem Tissue *

Fraction	Oxygen uptake		
	Without substrate	+ PPDA ** 0.045 M	+ Succinate 0.02 M
Sections	330	...	...
Residue	63.5	75	72.5
Homogenate	132	197	163
Washed mitochondria	−5.5	133.5	104.5

* The respiration of intact tissue sections of pea stem internodes is compared to the oxygen uptake of the residue after extraction, the homogenate, and the washed mitochondrial preparation, all in the same experiment. Oxygen uptake is given as microliters per gm original fresh weight per hr, Q_{O_2} (FW).

** PPDA = *p*-phenylenediamine.

so completely extracted. In one experiment summarized in table I, the oxygen uptake of the residue was compared with that of intact sections, a homogenate, and washed particles. It is seen that the residue has about one fifth the respiration of the original sections. It may be reasoned that the respiration of the residue must be due either to intact cells, corresponding to tissue sections; broken cells, corresponding to a homogenate; or trapped particles, corresponding to washed particles (M_w). Other experiments had shown that intact sections were not stimulated more than 20 to 25 % by added *p*-phenylenediamine or succinate. An examination of table I suggests, therefore, that the respiration of the residue is due largely to unbroken cells. This is in partial support of the assumption of Millerd (22). Duplicate experiments indicated that the residue accounted for an average of roughly 20 % of the respiration of the intact tissue. It may be assumed in consequence that the dehydrogenase activity of pea stem homogenates is approximately 80 % of that available in the intact tissue.

TABLE II

COMPARISON OF DEHYDROGENASE ACTIVITY AND RESPIRATION *

EXPT. NO.	TISSUE	RESP. ORS.	THEOR. REQUIREMENT FOR EACH ENZYME	ACTIVITY FOUND		
				SUCCINIC DEHYDROGENASE	MALIC DEHYDROGENASE	
					DPN 10^{-4}	DPN 10^{-3}
1	Avena	571	95	133	...	1140
2	Avena	566	93	146	465	915
3	Pisum **	370.5	62	68.5	453	684
4	Pisum **	373	62	76.5	625	866

* Dehydrogenases determined by rate of reduction of 2,6-dichlorophenolindophenol, by homogenates prepared as in text. Rates given as equivalent oxygen uptake.
** Pisum dehydrogenase values multiplied by 1.25 to allow for incomplete extraction.

SUCCINIC AND MALIC DEHYDROGENASE AND RESPIRATION: The Thunberg method, as adapted to the determination in plants of the dehydrogenases for succinate and malate, is very sensitive and fairly accurate, according to a number of criteria. Since the dilution employed is considerable (10 to 50 mg FW per 4 ml of test solution), it is possible to assay homogenates of substrate-rich tissues without encountering significant blanks.

The results of several experiments, comparing the dehydrogenase activities and the rates of respiration of tissue aliquots, are summarized in table II. The results, expressed in microliters of oxygen uptake per hr per gm FW, show that the succinic dehydrogenase activity of the oat coleoptile can consume oxygen at a rate equal to about one quarter of the rate of respiration. For the pea stem it corresponds to about one fifth the rate. The maximal malic dehydrogenase activity, with coenzyme I nearly saturating, corresponds to about twice the entire rate of respiration. Since the decarboxylating "malic enzyme" requires coenzyme II, its activity is not included in this measurement.

Thus, the succinic dehydrogenase activity is slightly more than theoretical for operation of the Cycle, while malic dehydrogenase has many times more activity than is required.

ALPHA-KETOGLUTARIC, SUCCINIC, AND MALIC "OXIDASES": Dehydrogenase activity can also be measured by means of oxygen uptake, whereby the dehydrogenase is linked, through the endogenous cytochrome system, to oxygen ("oxidase"). In the case of plant α-ketoglutaric dehydrogenase, this is the only means thus far developed for its detection. As discussed earlier (25), the enzyme specificity is lower with oxygen than with dye reduction since the initial rate of oxygen uptake corresponds to secondary oxidations of the reaction product. It is possible, nonetheless, to estimate, by means of empirically determined factors, the approximate rate of dehydrogenation of the organic acid from the measured oxygen uptake.

In the simplest case, the entire oxygen uptake would be due to a single-step oxidation. This is true for the initial stage of malate oxidation, in which direct analysis shows that a mole of malate disappears per atom of oxygen used. With succinate, the corresponding factor was found to be 0.9. With α-ketoglutarate, however, the factor was only 0.5 (24) which means that half the oxygen uptake is due to oxidations beyond succinate. Hence the organic acid oxidized may be approximated by multiplying the Q_{O_2} values by 1.0, 0.9 and 0.5 for malate, succinate and α-ketoglutarate, respectively. In order to allow for incomplete extraction, each factor must be further multiplied by 1.25 when comparing the enzyme activity to the rate of respiration in pea stems.

With plants there is a further restriction on the manometric method, namely that the tissue homogenates are too dilute to give accurate estimations; as

TABLE III

COMPARISON OF "OXIDASE" ACTIVITY OF WASHED MITOCHONDRIAL SUSPENSIONS WITH RESPIRATION OF PEA STEM INTERNODES *

	α-KETOGLUTARIC OXIDASE		SUCCINIC OXIDASE		MALIC OXIDASE		RESP.
	Q_{O_2} (FW)	Q_{O_2} (N)	Q_{O_2} (FW)	Q_{O_2} (N)	Q_{O_2} (FW)	Q_{O_2} (N)	Q_{O_2} (FW)
	85.0	...	102	...	120	...	421
	44.5	295	82	525	78	498	271
	49.5	317	126	800	106	678	299
Ave.	60		103		101		330
Factor **	0.625		1.125		1.25		...
Q_{O_2} (ave.) × factor † ..	37		116		126		55 ††
% theoretical	67		210		229		...

* Separate rows refer to averaged values from separate experiments.
** $\frac{\text{Equivalents organic acid}}{\text{Equivalents } O_2} \times 1.25$. For derivation see text.
† Rate of organic acid disappearance expressed as equivalent oxygen uptake.
†† Theoretical = ½ respiration.

a result, mitochondrial or other particulate suspensions, which can be concentrated from a large amount of tissue, must be employed. But unfortunately, many enzymes are only partially included in these fractions (11, 25, 28), and hence the values will in many cases be low. In addition, the manometric method is insufficiently sensitive for small amounts of plant tissue; in the case of the oat coleoptile, for example, more than 100 ten-mm sections are desirable for a single vessel.

These limitations must be borne in mind in making comparisons of the respiration and "oxidase" activity of pea stem tissue, as summarized in table III. Nevertheless, it is evident that the oxygen consumption of the mitochondrial fraction agrees moderately well with the theoretical levels required for the cycle. Only α-ketoglutarate is oxidized at a rate lower than theoretical, its value being one-third too low. Since the total oxygen uptake with malate is greater than with α-ketoglutarate, a limiting element not common to the two "oxidases" is indicated. The addition of a crude coenzyme A preparation did not stimulate oxygen uptake. The dehydrogenase itself appears therefore to be limiting in the electron transfer chain and the assay is correspondingly validated. However, in view of the considerable reservations attendant to this method, it might be predicted that a direct assay measuring α-ketoglutaric dehydrogenase of the whole homogenate would show activity equivalent to or exceeding theoretical.

TABLE IV

COMPARISON OF DEHYDROGENASE AND "OXIDASE" ACTIVITY *

SUBSTRATE	DEHYDROGENASE	"OXIDASE"
Succinate	232	342
	294	395
Malate + 10^{-4} M DPN	...	332
	302	409
Malate + 10^{-3} M DPN	995	445
	1075	483

* Single preparation of washed mitochondria in Thunberg and manometric tests; except for particle dilution and terminal oxidant, identical conditions throughout: 0.02 M substrate and 0.01 M phosphate, pH 7. Oxygen uptake calculated as Q_{O_2} (N). The sets of data are from two separate experiments.

CORRELATION OF DEHYDROGENASE AND "OXIDASE" DETERMINATIONS: It is clearly important to determine what correlation can be established between the two methods of dehydrogenase analysis. For this purpose a mitochondrial preparation, suitable for manometric estimation, was tested with succinate and malate with both the dye and oxygen as hydrogen acceptors and under identical conditions of substrate concentration, pH, etc., the only difference being the lower enzyme concentration in the former.

Table IV shows that with succinic dehydrogenase the agreement, while not perfect, is acceptable. Indeed, considering the 30-fold difference in enzyme concentration as well as the difference in the hydrogen acceptors, the correlation is quite good.

In the case of malic dehydrogenase, agreement is obtained only at low coenzyme concentration. In manometric experiments (20, 25) there is considerable activity in the absence of added coenzyme, but additions of it do not lead to as high values as with the dye as acceptor. It would appear, therefore, that a step between flavoprotein and oxygen is limiting.

DISCUSSION

Some years ago the comparison of rates of respiration and enzyme activity excited great interest (see the review of Krebs (17)), but in the animal tissues studied, it is extremely difficult to estimate the maximum level of respiration of an intact organ or tissue. The problem with plants is much more straightforward, since tissues are easily excised with a minimum of damage and are presumably less subject to a complex array of hormones. Nonetheless, few such studies have been undertaken (13, 14, 22); only one of these, that of Millerd (22), purports to deal with a comparison of the Krebs cycle enzymes and respiration, as does the present study.

In Millerd's report the oxygen uptake of a mitochondrial preparation oxidizing succinate (45 μl/hr $\times$ gm FW) is considered to account for the entire respiration of the intact tissue (150 μl/hr $\times$ gm FW), by assuming only 50 % efficiency of extraction and 50 % loss during centrifugation. These assumed losses were based on visual observation and do not correspond to our data on extraction, given above, nor to our data on recovery from centrifugation (25), although these were obtained with very similar tissue.

However, Millerd assumed that all of the oxygen uptake with succinate represented complete oxidation through the cycle. This was not proven, and indeed, the oxygen uptake in the presence of succinate, both of plant particles (25) and of muscle homogenates (table 3 of ref. 19) is due almost entirely to the oxidation of succinate itself and not of other cycle intermediates. As a result, the theoretical requirement for succinic oxidase is much lower than it would be for cyclic oxidation. Considerably larger respiratory rates and enzyme activities were found in the present paper (tables III and IV) and one may conclude that the plant tissues studied can oxidize succinate at a rate greater than one sixth of the oxygen uptake of the intact tissue. With a tissue RQ of 1.0 or less the succinic dehydrogenase activity thus equals or exceeds the theoretical level for Krebs cycle respiration.

A similar argument can be made for malic dehydrogenase. The total malic dehydrogenase, and even that fraction of it associated with the mitochondria which can be coupled with the oxidase, appears sufficient for the purpose. In view of the apparent excess of malic dehydrogenase, it may be suggested that the 75 % of this enzyme appearing in the soluble fraction (25) has a separate function. "Malic enzyme" may presumably be ruled out, since it is specific for coenzyme II.

α-Ketoglutaric acid oxidation appears to have the most complex requirements of those tested; it is unlikely therefore that truly optimal conditions have been established. In fact considerable variability in the Q_{O_2} (N) values has been observed, many single values appearing to be higher than those reported in table III. It is possible, therefore, that the activity found in these experiments is on the low side and that the true α-ketoglutaric dehydrogenase of the particulate fraction is sufficient for the operation of the cycle. However, it must be recalled that α-ketoglutaric acid was one of the least active acids in promoting growth of isolated coleoptiles (see (6) table 2). This behavior certainly suggests that its oxidizing enzyme might be a limiting factor.

Although it is quite possible that the enzyme activities in vivo may be higher than we have been able to demonstrate in vitro, there are several considerations that suggest that the in vivo activities are not maximal. When more than one substrate is added to a particulate preparation, the rate of oxidation is not additive, indicating competition for pathways of electron transport. Furthermore, there is mutual inhibition among the cycle intermediates. With malate oxidation these effects are very pronounced.

Of the two other dehydrogenases of the cycle, little of a quantitative nature can be said. Iso-citric dehydrogenase appears to be fairly active in plant extracts, but pyruvate oxidation has been found so far to go only slowly (12, 21, 22).

From the present study it can be concluded that of the five dehydrogenases required for respiration through the Krebs cycle, two are present in adequate amounts and a third one may perhaps be. This is affirmative evidence, within its limitations, for the cycle as the main route of respiration in the plant.

Summary

The maximum activities of succinic and malic dehydrogenase, and of succinic, malic and α-ketoglutaric "oxidases" have been determined as accurately as possible in Avena coleoptiles and Pisum stem sections. These activities have then been compared with the observed maximum rates of respiration of the intact tissues, in order to determine whether the Krebs cycle is the main route of respiration or not. The limitations within which such comparisons are valid have been discussed. It is concluded that the amounts of succinic and malic dehydrogenase are sufficient to allow for all of the tissue respiration to proceed through them; in the case of α-ketoglutaric acid the amount of enzyme appears a little short of the theoretical. In the absence of information on the other dehydrogenations of the cycle, the evidence for the Krebs cycle as a main respiratory pathway remains incomplete.

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THE CARBON DIOXIDE CONTENT OF FIELD AIR[1,2]

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Studies of the CO_2 content of the air have been published over the last 200 years. Stepanova (20) gives 229 abstracts in her 1952 review, without including several extensive studies published in botanical journals or any work in which CO_2 measurements were incidental to studies of photosynthesis, soil respiration, etc. Although DeSaussure (7) showed diurnal variations in the CO_2 content of field air as early as 1816, and correctly interpreted them as due principally to photosynthesis and respiration, one 1952 paper is listed as an endeavor to clarify the disputed point of whether the CO_2 content of the air is constant or variable.

The point is of more than academic interest, for Böhning (3), Chapman and Loomis (5), Decker (6) and Thomas and Hill (21) have shown that photosynthesis varies directly with the CO_2 concentration at levels present in the field. Normal CO_2 is generally considered to be 0.03 volume percent, which is 300 ppm or 0.594 mg/liter of air at sea level and 0° C. Glueckauf (9) believes, however, that the average concentration is increasing with the utilization of oil and coal, and is now above 330 ppm. Balloon and rocket flights have shown that the volume percentage of CO_2 is essentially uniform to heights of at least 70 km or 42 miles (9). A value of $0.029 \pm .002$ % at 72,000 ft was reported by the Explorer II ascent (17). Atmospheric pressure at this altitude is about 5 % of that at sea level and the quantity of CO_2 per unit space is correspondingly low, but the volume percentage is unchanged (23).

Verduin and Loomis (24) reported reductions in the CO_2 content of field air during rapid photosynthesis in Iowa averaging 25 % of the "normal" concentrations. Similar changes have been reported recently by other workers (4, 11, 15, 16, 22, 25). Chapman (4) found less fluctuation in the CO_2 content of the air in a dryland field at Alliance, in western Nebraska, than had been reported for Ames, Iowa. He assumed that the lower soil organic matter, moisture and temperature reduced the build-up of free CO_2 at night, and that the generally more sparse vegetation resulted in less utilization during the day. Results in Palestine (16) were similar to those in Iowa, but analyses at Milano, Italy (22) showed a considerable increase in average CO_2 content of the air in the summer. The daytime drop during the summer was of the same magnitude as that in Iowa.

Verduin and Loomis (24) noted that the low daytime CO_2 at ground level was not increased appreciably by winds of moderate velocity, thus raising the question of stratification and limited mixing of surface air with higher levels. In the summer of 1952 the opportunity arose of using the Iowa State College television tower for continuous sampling of air at a moderate height in comparison with analyses from an adjoining corn field.

Methods

A modification of Heinicke's method (10) described by Chapman and Loomis (5) was used. Five lines of 3/16 inch I.D. copper tubing were connected to CO_2 absorption towers. These lines sampled the air at: (a) 1 m above the ground and about 20 m inside the border of a large corn field; (b) 1 m in the TV tower area, a grass plot about 40 by 60 m completely surrounded by corn; (c) 10 m above the ground on the tower; (d) 30 m on the tower, and (e) 152 m at the base of the transmitting antenna on the tower. Manometers and thermometers were inserted in the air lines just ahead of the flow meters to provide data for correcting apparent air volumes. Temperature differences were small; pressure drops due to line resistance varied between about 10 and 50 cm of water.

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[2] Journal paper No. J-2521 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project 1139.
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Equal volumes of air were drawn from each station at the rate of 100 l/hr and CO_2 was absorbed in standard KOH in towers which gave better than 99 % absorption at the rate of flow used. Carbonates were precipitated from the residual KOH with $BaCl_2$ and the solution titrated to the phenolphthalein endpoint with HCl. Carbon dioxide was then calculated as mg/l at standard pressure and temperature.

Results

Experiments were started in July when the corn field was tasseled and continued at intervals until October 18 when all corn plants in the area were dead and dry. Typical runs started at 3:00 A.M. and continued until midnight. Various shorter runs were made to test morning and evening changes, effect of wind, etc. Figure 1 shows the type of data obtained on still days with a maximum wind velocity of 2 to 3 mph. Analyses at 1 m in the grass plot around the tower were generally not significantly different from those in the corn field. Results at 10 and 30 m on the tower tended to be intermediate between those at the lower and higher levels, with figures at 30 m rather close to those at 152.

The curves of figure 1 show a build-up of CO_2 near the ground at night. This build-up is due to respiration of plants and soil organisms (13, 18) and develops only on a still night. It did not show at 30 m or above until after sunup, when rising currents of warm air may have caused the sudden jump of CO_2 content shown at 6 A.M. in figure 1. Fuller's results (8) suggest that a still higher concentration might have been obtained by sampling the air just above ground level. Fuller made his measurements in natural forest or prairie vegetation, however, where respiration of soil organisms and trapping of CO_2 by low vegetation would have been important. Our measurements were made in a well cultivated corn field with dry surface soil and we suspect that plant respiration was more important than soil reactions in the data obtained. The daytime drop shown in figure 1 amounted to nearly 40 % in three hours, and the low point, just after noon, was about 25 % below "normal." Several other points are worthy of note. The CO_2 percentage at 152 m was uniform between 8:00 A.M. and 9:00 P.M. at 90 % of normal. The average CO_2 content in the field was down another 10 %, but the values fluctuated more. Verduin and Loomis (24) found concentrations during the middle of the day to be nearly uniform at ground level, but many of the curves in this series of experiments showed fluctuations. The results suggest more turbulence than was encountered previously, although less would have been expected from the terrain. Note also in figure 1 the slow mixing between the two levels in the evening before the wind arose, contrasted to the rapid changes of the upper level in the morning when thermal currents were present.

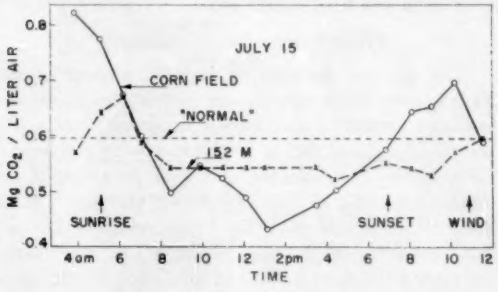

Fig. 1. Variations in the CO_2 content of air in a corn field and 152 m above it on a still day. A CO_2 deficit of more than 100 lbs an acre was developed within 3 hrs after sunrise, to remain nearly constant until late afternoon.

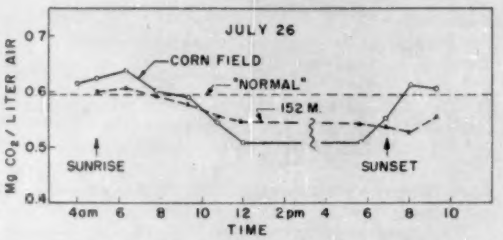

Fig. 2. Variations in the CO_2 content in a corn field and 152 m above it on a day with winds of 3 to 8 mph. Changes were less abrupt than those shown in figure 1, but very considerable deficits were still present.

July 15 was an almost windless day and the amount of stratification of CO_2, both at night and in the day, was unusually high. The curves of figure 2 show the changes on a day with a steady wind of 3 mph morning and evening and 8 mph during the day. The same pattern is evident as in figure 1 but changes were smaller and more gradual. CO_2 on the ground did not build up much above normal at night; the morning drop was much more gradual, and there were fewer fluctuations, although readings in the afternoon might have shown more of these. Depletion of CO_2 at the ground level was about 13 %, but depletion at 152 m was again 9 or 10 %; also the evening pattern of a rapid rise at the low level and a slower one on the tower was repeated.

The curves of figure 3 A show the picture obtained a week later with a wind of about 8 mph after 4:00 A.M. and 5 mph before. The night-time build-up in the field was absent, and there was little difference in CO_2 concentrations at the field and tower levels. It is interesting to note that the minimum CO_2 concentration recorded at both ground and tower levels on the windy day was near that found on the tower in the previous runs; a decrease of 10 to 12 % below normal.

The curves of figure 3 B show the results obtained after frost. All corn was mature at this time and only the reduced rate of photosynthesis in frosted grasses and other hardy plants was affecting the local daytime CO_2 level. The differences shown are erratic and in part non-significant. The dip at 8:00 to 9:00 A.M. may be the result of air moving in from greener

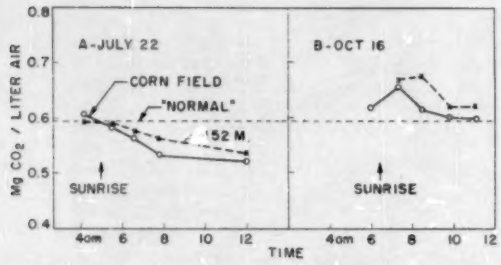

FIG. 3. A. CO_2 variation on a windy morning—similar to figure 2. B. CO_2 variation after frost. Note higher concentrations and erratic changes in the absence of important amounts of photosynthesis.

areas to the southwest on a 10 mph wind. On October 18 the CO_2 content of air from all levels dropped nearly 20 % between 10:00 and 11:00 A.M. under similar conditions. In none of these readings, however, did the CO_2 drop below "normal." With or without wind, these late readings showed closely agreeing values at all levels.

CO_2 Balance

The CO_2 content of air at the high level was always near normal in the morning, indicating that the CO_2 deficits of the previous day had been made up—by movement of CO_2 from higher levels, and by respiration of plants and soil organisms. Bair (1) has shown that an Iowa corn crop is produced at a uniform daily rate, some 90 % of it between about July 1 and September 1. CO_2 consumption by corn should have run 200 lbs or more per acre per day with the yields obtained in 1952. Maximum CO_2 absorption by this crop is more than 400 lbs per day. Allowing for lower rates in stubble and crops other than corn, we may consider that the average CO_2 consumption at the time the July readings were made was in the neighborhood of 125 lbs/acre × day, equivalent to 8000 or 9000 lbs/acre × crop year. Bartholomew (2) estimates that CO_2 production by central Iowa soils is about 2200 lbs/acre × year from the high original organic matter, plus the CO_2 equivalent of all crop residues. For corn the residue CO_2 would be 6000 to 8000 lbs per year. Lower production by other crops should be balanced by combustion of fuel and animal respiration. The latter is a not inconsiderable item in Iowa where 90 % of the corn grain produced is fed to livestock within the state. An annual CO_2 balance is thus indicated without any considerable exchange with the huge CO_2 reservoir of the ocean (12).

Temporary unbalance during the day is indicated by the curves of figures 1 to 3. The night-time accumulation shown in figure 1 was measurable at 10 but not at 30 meters and a total accumulation of only 11 lbs/acre is estimated to have occurred, even with one of the highest CO_2 readings we have obtained. A reduced loss of CO_2 from the soil is expected at night because of reduced temperatures and particularly because of the downward movement of air into the cooling soil. In contrast to the negligible accumulation, a deficit of at least 100 lbs/acre and probably 150 lbs was built up during the day; a large part of it by 8:00 A.M. Even on the windy days (figs 2, 3 A) a deficit of 70 to 100 lbs/acre was observed. Actually the deficits could have been considerably more, since the type of thermal mixing, assumed to be responsible for the 6:00 A.M. jump of CO_2 on the tower (fig 1) may be felt as a decided bump in a light plane at 2000 ft, and under favorable conditions the CO_2 deficit may be assumed to extend in diminishing value to this or greater heights. Since our data are corrected to standard temperature and pressure, expansion of the air cannot account for the decrease, and no ready explanation is apparent unless the rates of photosynthesis on these days were considerably above calculations.

We have indicated that the total annual production of CO_2 seems to be in balance with use in central Iowa. The daily production of CO_2 by respiration of soil organisms varies markedly, however, with temperature, soil organic matter, and particularly with soil moisture. Lundegårdh (14) measured soil respiration rates of 0.1 to 2.12 gm/m^2 × hr, equal to 20 to 440 lbs/acre × day. Smith and Brown (18, 19) observed CO_2 production rates of 0.15 to 2.72 gm/m^2 × hr in a series of plots when the soil was moist, and rates of 0.016 to 0.19 gm/m^2 × hr in the same plots during dry weather. The lowest rate is about 3 lbs/acre × day. We have made no CO_2 measurements during pronounced drought, although the soil was dry on the surface during most of the time our records were taken. Since air at higher levels may move 1000 miles or more a day, we must assume that even local CO_2 supplies are affected by exchanges within the total reserve, and that deficits built up during the day would tend to even out with time. The rapid recovery on still nights is probably heavily dependent, however, upon redistribution of the 40,000 lbs of CO_2 normally present in the air over each acre (12), and the rather uniform daytime deficit at 152 m probably represents the level at which movement from higher altitudes balances the daytime excess of photosynthesis over respiration.

Discussion and Summary

The daytime decrease in the CO_2 content of the air among plants carrying on active photosynthesis has been confirmed and shown to extend, with some lag, to a height of 152 m or 500 ft. A typical fluctuation in the CO_2 content at 152 m was 0.03 % at night, decreasing to 0.027 % during the day. With winds of 5 mph or more the CO_2 content of the air in a corn field sampled at a height of 1 m, was nearly the same as that at the 152 m level, but on still days and nights a maximum variation of 0.02 % has been observed; from 0.041 % at night to 0.021 % during active photosynthesis. Since the equilibrium CO_2 level at which net photosynthesis becomes zero is in the neighborhood of 0.01 % (21) such marked drops

in the CO_2 content of the air may be expected to limit photosynthesis and crop yields.

The estimated annual per acre production of CO_2 in Iowa is of the same order as its use in photosynthesis, with decay of organic matter in the soil, or respiration of soil organisms, the dominant factor in CO_2 production. Although daily production may be considerably more or less than use, the 40,000 lb reserve of the air, plus exchange with other regions by air currents, serves to bring the CO_2 supply in the air a few meters above the soil to near "normal" each night. A deficit of 10 % in CO_2 at 150 m during rapid photosynthesis may represent the gradient across which excess use by plants can be replaced from higher levels of the atmosphere.

The rapid fluctuations in the CO_2 content of field air indicate that attempts to measure variations in different growing seasons in the average CO_2 content of the air (14) are subject to large errors and the results difficult to interpret. Similar problems beset attempts to measure long-time changes in average CO_2 concentration (9).

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A DEVELOPMENTAL STUDY OF D-GLYCERIC ACID DEHYDROGENASE[1,2]

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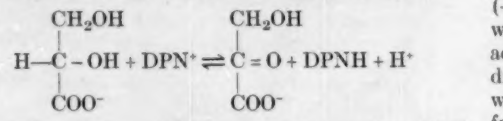
A previous publication from this laboratory (13) presented evidence for the occurrence in higher plants of a new enzyme, D-glyceric acid dehydrogenase, which catalyzes the reversible oxidation of D-glycerate to hydroxypyruvate in the presence of diphosphopyridine nucleotide[5] according to the following equation:



Since the mere presence of an enzyme in a tissue does not establish a physiological role, further data are needed in order to elucidate the possible metabolic function of this enzyme. As an aid in obtaining such information, the developmental pattern of this dehydrogenase was studied. The previous investigation (13) had shown that although D-glyceric acid dehydrogenase is widely distributed in the tissues of various higher plants, the enzyme is present in a particularly high concentration in green leaves. This localization has been substantiated by following the pattern of the increase in the enzymatic activity upon germination. Another DPN-dependent enzyme, glyoxylic acid reductase (17), was studied for comparison. Peas and wheat were selected as representative plants because of their ease of growth under laboratory conditions.

MATERIALS AND METHODS

Pea seeds (*Pisum sativum* var. Alaska) or wheat seeds (*Triticum aestivum* var. Pawnee) were sterilized for 15 minutes with a saturated solution of calcium hypochlorite before being soaked for 2 hours in distilled water. The seeds were placed on a layer of cheesecloth supported by a wire frame that was set into enamel trays containing sufficient nutrient solution to keep the cloth moist. The nutrient solution contained 0.005 M $Ca(NO_3)_2$ and KNO_3; 0.001 M $MgSO_4$ and KH_2PO_4; 2.5×10^{-4} M K_2HPO_4; and 1.8×10^{-5} M $FeSO_4$. After being covered for the first 2 to 3 days, the trays were placed at room temperature under several white fluorescent lamps (about 300 to 500 fc of light) for 8 to 10 hours a day. These plants are called "light-grown." Plants grown under comparable conditions except for being kept in complete darkness are referred to as "dark-grown" plants. Good growth was maintained under these conditions.

The plants were harvested at intervals and approximately 10 to 20 grams fresh weight of the portions to be analyzed were weighed, washed and then ground at 4° C for 1 minute in 200 ml of cold (−18° C) acetone in a Waring blendor. The powder was filtered and washed with another 200 ml of cold acetone in successive small portions, and then further dried and stored at 4° C in a desiccator under vacuum with $CaCl_2$ as desiccant. This powder was extracted for 0.5 hour at 4° C in 25 to 50 ml of 0.001 M phosphate buffer (pH 7.4), filtered through glass wool, and then centrifuged at high speed to give a clear supernatant. Dry weight analyses were made on this final extract. Dialysis made no appreciable difference in the enzymatic activity and was omitted. These acetone powder preparations gave a greater total activity for glyceric acid dehydrogenase than dialyzed extracts made by grinding frozen leaves in a mortar. The ratio of the activity of glyceric acid dehydrogenase to glyoxylic acid reductase was similar in the two different preparations.

Since the equilibrium of this dehydrogenase reaction lies far in the direction of glyceric acid and DPN$^+$, the enzymatic activity is most easily measured by observing the oxidation of DPNH in the presence of hydroxypyruvate. Therefore, in the subsequent experiments, the oxidation of DPNH was followed in a Beckman spectrophotometer by observing the change in the optical density (log I_o/I) at 340 mμ. Because DPNH is oxidized in the absence of added substrate by some of the enzyme extracts used (2), a control mixture without added substrate was always assayed in each experiment, and this rate of oxidation of DPNH was subtracted from the rate measured in a mixture with added substrate.

Suitable aliquots of the enzyme extract were added to each experimental cuvette to maintain a constant change in optical densnty equal to or less than 0.020 per minute over a period of 10 minutes. The final rates are expressed as the change in optical density per minute per shoot or per mg dry weight of the enzyme extract. Besides the enzyme, the experimental cuvettes contained the following components in a total volume of 3 ml: 0.2 ml of an 0.5 M phosphate buffer of pH 6.5, 5 micromoles of either hydroxypyruvate, pyruvate, or glyoxylate, 0.2 micromoles of DPNH. These reaction mixtures were read against a solution containing the complete system minus DPNH. Increasing the concentration of substrate or of DPNH did not alter the rates of enzymatic activity.

The hydroxypyruvate, kindly supplied by Dr. W. Fager, was prepared from pyruvic acid according to Sprinson and Chargaff (12). The glyoxylate bisulfite

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[5] DPN$^+$ = oxidized diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide.

addition product, a gift of Dr. I. Zelitch, was prepared from oxalic acid according to Weinhouse and Friedman (16), and was hydrolyzed with HCl before being used. The DPNH was enzymatically reduced and isolated according to Loewus et al (7).

Reproducibility of the actual spectrophotometric measurements was limited only by pipetting errors, and therefore, caused little variation in the data. However, the data obtained from this developmental study does show variation. Some of this can be attributed to uncontrolled temperature conditions during the two separate growth experiments, and some to the necessity of obtaining acetone powders in order to remove pigments obscuring the spectrophotometric assay method. The rates therefore should be considered only as minimum values for each chronological age.

Results

Distribution of D-Glyceric Acid Dehydrogenase Activity within the Pea Plant: Extracts from dormant pea seeds contain a measurable, but weak D-glyceric acid dehydrogenase activity (fig. 1, A). When extracts from 11-day-old pea plants were assayed for the dehydrogenase, approximately 74 % of the total activity of the root shoot axis (minus cotyledons) was contributed by the leaves (leaflets, stipules and tendrils), about 20 % by the stems and the remaining 6 % by the roots. Similar results were obtained with 14-day-old plants. Therefore, although the enzyme is present in seeds and roots, the major portion of the activity is found in the leaves.

Developmental Study: If an enzyme is found predominantly in leaf tissue, a developmental study should show that the increase in the enzymatic activity is correlated with the growth of the leaves. Data from such a study using pea and wheat plants are shown in figure 1, A and B. The two separate plantings are indicated by solid and open circles and each point on the graph represents a single enzymatic assay. The enzymatic activity is expressed as the change in optical density per minute. The solid lines represent the activity per shoot, while the dashed lines represent the activity per mg dry weight of the enzyme extract. Approximate wet weight data per shoot and the height of the shoots are given in table I. While there is considerable variation between the 2 separate growth experiments, the trend is similar in both cases, and the following conclusions may be drawn.

Upon germination, the increase in dehydrogenase activity is slow until the shoot begins the period of maximum growth in length associated with rapid leaf growth. The enzymatic activity in both peas and wheat increases rapidly 4 to 10 days after germination, reaching a maximum after about 10 days of growth. This increase is closely correlated with the formation of new compound leaves in the pea plant (table I). The average increase per shoot during the 4- to 10-day-old period is equivalent to a change in optical density of 1.8 per minute in peas and 0.6 in wheat. The increase in activity per mg dry weight of the enzyme extract is less dramatic, especially in the case of peas.

After the peak value at 9 to 10 days, wheat plants show a significant decrease in activity, especially when the results are expressed as the activity per shoot. This decrease is not due to the presence of any inhibitor in the preparations as no inhibition was observed when a 14-day-old wheat extract was added to the more active earlier extracts of either wheat or peas. It is still possible, however, that the enzyme is inactivated in these older plants by a substance that is no longer inhibitory in the enzyme extracts. The decrease on a dry weight basis may mean that the synthesis of the enzyme is not keeping pace with the synthesis of other protein after this peak period at 9 to 10 days. The actual loss of activity per shoot is much less apparent or perhaps not existent in pea seedlings during the 14-day period studied, but the activity per mg dry weight decreases as in wheat plants.

All of the above preparations were assayed for lactic acid dehydrogenase because this enzyme can also reduce hydroxypyruvate (9). In the root and seed preparations, DPNH was oxidized upon the addition of pyruvate as substrate, but all of these preparations were unable to reduce DPN^+ in the presence

Table I

Growth Data and the Ratio of Glyceric Dehydrogenase to Glyoxylic Reductase Activity

Peas	Age in days						
	0	4.5	6.5	8	10	12	14
Height of shoot (cm)	...	1.5–2	3–5	11–15	14–18	20–22	24–27
No. cmpd. leaves	...	1	1.5	3–4	5	5	5
Gm wet wt per shoot	...	0.05	0.11	0.32	0.41	0.66	0.70
Glyceric/glyoxylic activity	3.5	6.5	8	8	9	13	14
	Age in days						
Wheat	0	4	5	7	9	12	14
Ave. height of shoot (cm)	...	5	7.5	14.5	16	22	26
Gm wet wt per shoot	...	0.03	0.05	0.10	0.12	0.15	0.19
Glyceric/glyoxylic activity	1	15	25	25	24	18	20

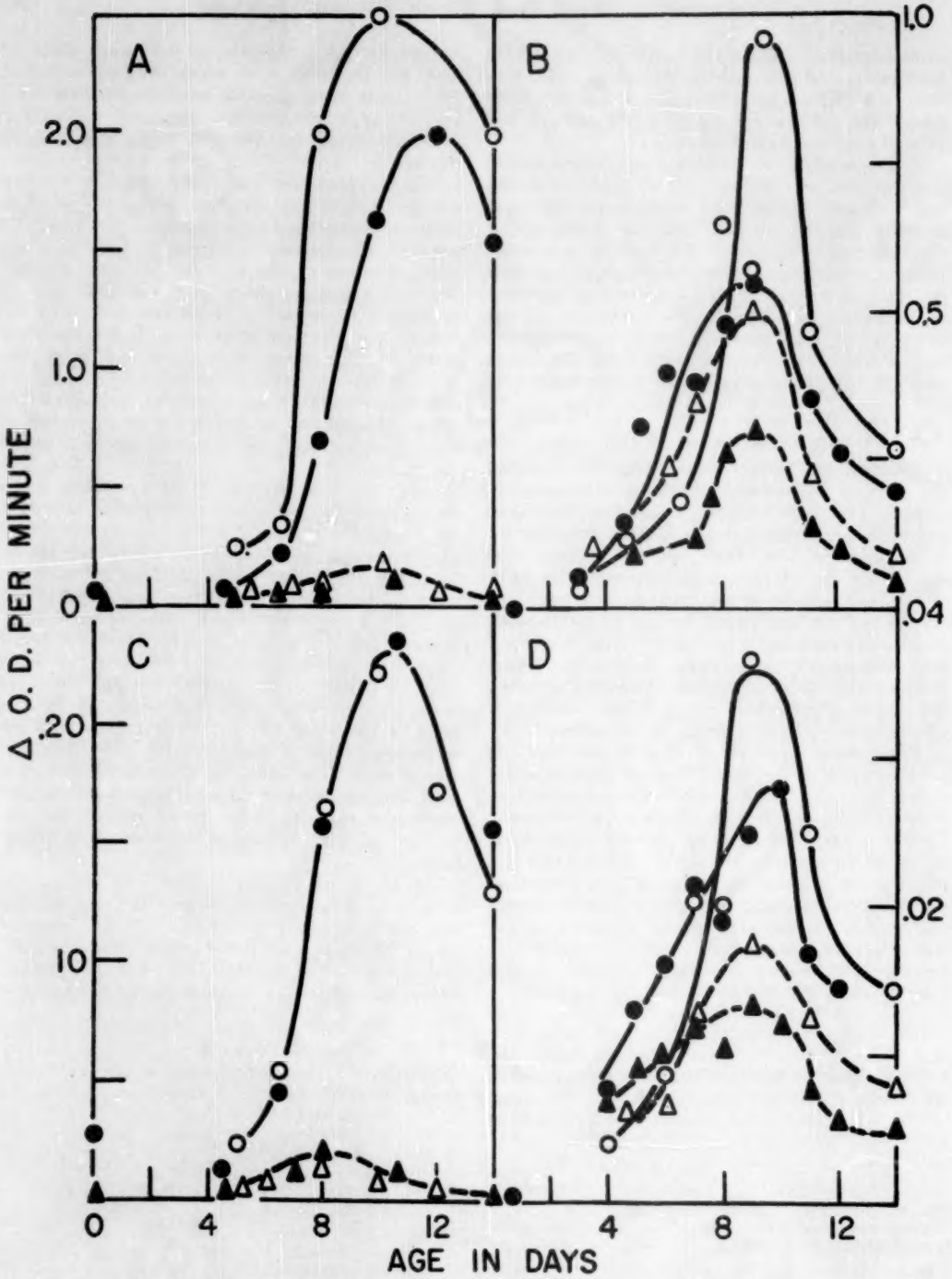

FIG. 1. D-Glyceric dehydrogenase and glyoxylic acid reductase activity in pea and wheat shoots. Graphs A and C show the enzymatic activity of D-glyceric dehydrogenase (A) and glyoxylic acid reductase (C) in peas. Graphs B and D show similar data for D-glyceric acid dehydrogenase (B) and glyoxylic acid reductase (D) in wheat. The enzymatic activity is equal to the change in optical density per minute either per shoot (circles) or per mg dry weight of the enzyme extract (triangles). Open and solid symbols represent separate growth experiments.

of lactate at pH 9, indicating the absence of a lactic dehydrogenase. The activity with pyruvate could be explained by the presence of pyruvic acid carboxylase, which would convert the pyruvate to acetaldehyde, coupled with alcohol dehydrogenase, which would then oxidize the DPNH.

Glyoxylic acid reductase activity of the same preparations follows a similar pattern of increase during the early growth of the shoot, but the actual rate of increase is not as rapid (fig. 1, C and D). For instance, the glyceric acid dehydrogenase activity of peas increases over 5 times as fast as that of glyoxylic acid reductase. The average increase per shoot from 4 to 10 days is equivalent to a change in optical density of 0.23 per minute in peas and 0.024 in wheat. This difference is partly reflected in a change in the ratio of the hydroxypyruvate to glyoxalate activity

TABLE II

EFFECT OF LIGHT ON THE ACTIVITY OF D-GLYCERIC ACID DEHYDROGENASE

		Dark-grown	Light-grown	
WHEAT		(9 DAYS—DARK 0 DAYS—LIGHT)	(8 DAYS—DARK 1 DAY—LIGHT) **	(0 DAYS—DARK 9 DAYS—LIGHT) †
Act.*/shoot	A	65	65	58
	B	42	28	..
Act./mg dry wt extract	A	23	28	29
	B	17	16	..
PEAS		(7 DAYS—DARK 0 DAYS—LIGHT)	(6 DAYS—DARK 1 DAY—LIGHT)	(0 DAYS—DARK 7 DAYS—LIGHT) †
Act./shoot		6	10	45
Act./mg dry wt extract		1	2	11

* Act. = change in OD (log I_0/I) per minute × 100.
** 24 hrs of continual light.
† 8–10 hrs of light per day.

from about 3 to 15 in peas, and from 1 to 25 in wheat (table I).

EFFECT OF LIGHT ON THE ENZYMATIC ACTIVITY: Since glycolic acid oxidase (14) and TPN-glyceraldehyde dehydrogenase (4) have been reported to be found predominantly only in light-grown plants, the effect of complete darkness during the growth of the seedlings on the enzymatic activity of D-glyceric acid dehydrogenase was investigated. In contrast to the above mentioned light-dependent enzymes, the activity of this dehydrogenase was equal in light- and dark-grown plants of similar size.

Except for some difference in total height, the pattern of growth in wheat is similar in plants grown for about a week in the dark or in the light. Two trays of wheat seeds were grown in the dark for eight days, and then one tray was removed and kept in continuous light for the next 24 hours. Little or no difference in activity was observed as shown in table II (A and B represent separate experiments). Comparable data obtained from plants placed in the light each day after germination are shown in the last column.

Although peas are not a satisfactory test material because the growth pattern is highly modified in the dark, with internodal rather than leaf growth predominating, similar results are obtained if one compares plants of the same size. Pea seedlings, grown for 6 days in the dark and subsequently placed in the light for 24 hours, had approximately the same activity as those left continually in the dark. In contrast to wheat plants, however, the amount of enzyme (and leaf development) was comparable to that of 4.5-day-old rather than to 7-day-old light-grown pea plants (table II).

INTRACELLULAR LOCALIZATION: Tolbert and Cohen (15) reported that 10 % of the total activity of glycolic acid oxidase of leaves was associated with a possible chloroplast fraction. They felt that this small percent was truly a part of the particulate complement because the activity was retained in a variety of buffers at different pH values.

Preliminary studies were made to indicate the intracellular localization of D-glyceric acid dehydrogenase in leaves. Crude particulate fractions were separated from soluble enzymes in the supernatant by centrifugation of sucrose-phosphate (8) or sorbitol-borate-KCl (3) homogenates of green pea shoots at 18,000 × g for 60 minutes. The centrifuged particles were washed once with the same medium, and then both fractions were precipitated with acetone. Approximately 5 to 10 % of the total activity (of the particles plus the supernatant) was associated with the washed crude particulate fraction (presumably containing chloroplasts, mitochondria, etc) while the remaining 90 to 95 % appeared in the soluble fraction of the supernatant. Although the present study with peas cannot differentiate between secondary adsorption during extraction, and the valid localization of this small percent of activity with leaf particles, the majority of the enzyme is certainly soluble under the conditions employed.

DISCUSSION

The metabolic function of the two organic acids, glyceric and hydroxypyruvic, are not well known. Glyceric acid is of interest because of its possible relationship to the early photosynthetic intermediate, 3-phospho-D-glycerate. Free glyceric acid has also been identified among the early products of photosynthesis (1), but it is not clear whether this is merely the result of phosphatase activity. In a study of the synthesis of ascorbic acid in cress seedlings, Isherwood, Chen, and Mapson (6) observed that an increase in D-glyceric acid was generally associated with the ascorbic acid increase which occurs during the first 5 days of growth after germination. Although the relationship between these two substrates was not elucidated, D-glyceric acid did not appear to be a precursor of ascorbic acid, nor was ascorbic acid the precursor of D-glyceric acid.

Regardless of the relationship between ascorbic and glyceric acids, it is significant that D-glyceric acid has been identified as a major acid constituent in a higher plant, and that the increase in glyceric acid content might coincide with the increase of the enzyme D-glyceric dehydrogenase. Although the present developmental study was not concerned with cress seedlings, water cress shoots (*Nasturtium officinale*) are an active source of the enzyme, giving a total change in optical density of 0.26 per minute per mg dry weight of an extract of an acetone powder. While Isherwood and coworkers suggest that the glyceric acid in cress seedlings could arise from the activity of 3-phosphoglycerate phosphatase or glycerol dehydrogenase, the D-glycerate could be due to the action of D-glyceric acid dehydrogenase upon hydroxypyruvate. This of course, necessitates the explanation of the origin of hydroxypyruvate within the plant.

Hydroxypyruvate has been isolated from extracts of mammalian tissues after incubation with serine (11). A similar isolation has not been made from plant tisues, but the recent investigations (5, 10) of the enzyme, transketolase, indicate that hydroxypyruvate can serve as a source of active aldehyde in the formation of pentose phosphate from triose phosphate.

Possible interrelationships between enzymes and substrates which increase upon germination or which are concentrated in leaf tissue need to be further elucidated.

Summary

D-Glyceric acid dehydrogenase is predominantly a leaf enzyme, although low activities are found in roots and seeds of wheat and peas. The main increase in enzymatic activity during the early growth of the shoot is associated with maximum leaf development. The enzyme is found in comparable amounts in dark- and light-grown plants of similar size and is predominantly a soluble enzyme. Comparisons are made with glyoxylic acid reductase, which shows a similar but smaller increase during the early growth of the shoot.

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PARTIAL REACTIONS IN THE FORMATION OF THE FLORAL STIMULUS IN XANTHIUM [1]

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The formation of the flower-inducing principle in the leaves of *Xanthium pensylvanicum* Wallr. has been shown by Hamner (1) to consist of at least two partial reactions: a preliminary high intensity light process, followed by a process requiring for its completion a minimum period of continuous darkness. Recently it was reported (3) that the flowering response of Xanthium may be suppressed when the inductive dark period is followed (after a brief interruption by light) by a second dark period of 4 to 6 hours duration. The effectiveness of the second dark period is greatly enhanced by treatment of the plants with indoleacetic acid (IAA). These results suggest that certain processes necessary for the formation of the flowering stimulus must take place in the leaf following an inductive dark period. Experiments were, therefore, conducted in an attempt to further characterize these processes.

The flower inducing principle has not been identified as a single chemical compound, therefore we prefer not to refer to it as a hormone. In this paper it will be referred to as an internal stimulus, which is formed in the leaves and moves to the terminal meristem, where it changes the environment of the growing point causing the differentiation of the floral inflorescence.

The results reported here will show that a photochemical process stabilizing the floral stimulus takes place after the inductive dark period and before the flowering stimulus is transported from the leaf. This process may be measured by a decrease in the effectiveness of the second dark period resulting from exposure of the leaves to light after termination of the inductive dark period. The photochemical reaction which stabilizes the stimulus requires high intensity light for approximately 5 hours and in the absence of such light treatment the stimulus remains sensitive to destruction by a second dark treatment for at least several hours.

GENERAL METHODS AND MATERIALS

The methods and materials employed were similar to those reported previously (3). All older leaves of the experimental plants were removed within 24 hours before treatment, leaving only the 2 youngest fully expanded leaves and the expanding leaves during treatment. The stage of flowering was determined by dissecting the plants 3 weeks after treatment. Stages were assigned according to the morphological development of the inflorescence, according to a method developed in this laboratory (6). The relative stages seem to reflect accurately the quantity or intensity of flowering stimulus reaching the terminal meristem. From 8 to 10 plants were used in each experiment and the differences found here were highly significant. All data reported are representative of two or more separate experiments giving similar results.

Low temperatures were obtained in refrigerated rooms maintained at $\pm$ 2° C of the desired temperature. In these rooms, cool white fluorescent lights were installed providing illumination of 1200 fc at the leaf surface. The temperature of the illuminated cold room was measured by hanging a mercury thermometer under the lights at the level of the leaves. Some difficulty was encountered in preventing wilting of the plants kept at 5° C in high intensity light in excess of 4- to 5-hour periods. In some cases where wilting did occur the subsequent flowering response was greatly decreased and certain treatments were discarded when it seemed clear that wilting critically interfered with the results obtained.

It has recently been found in this laboratory (2), that if the second dark period is given at elevated temperatures (40° C) the effectiveness of this dark period is greatly enhanced. The application of IAA, which was done in previous work (3) in order to attain maximum destruction of the stimulus, would have introduced undesirable complications in some cases. Therefore, in certain experiments, the plants were exposed to the second dark treatment, 3 hours darkness at a temperature of approximately 40° C. To give this treatment, an insulated room was equipped with heating units wired through a bimetallic thermoregulator and a mercury relay. A fan was used to circulate the air and the temperature was maintained at 39.5 $\pm$ 0.1° C. All data reported here are representative of two or more separate experiments giving similar results.

EXPERIMENTAL RESULTS

EXPERIMENT 1: A number of experiments have demonstrated that, following a 12-hour inductive dark period, if the plants are exposed to 10 minutes of light, treated with IAA and given a second dark period of 5 to 6 hours, the flowering response is reduced to a value of 0 to 20 % of the control plants. Experiments were undertaken to determine whether the stimulus remained sensitive to the IAA and second dark period treatment as long as it remained in the leaf. Plants were placed under fluorescent lights (1000 fc) immediately following a 12-hour inductive dark period and, at various time intervals thereafter, treated with IAA and given a second dark period of 5 hours. To determine whether the stimulus had moved out of the leaf, control plants were given the same dark induction, placed under the fluorescent lights and groups defoliated (detailed below) at the

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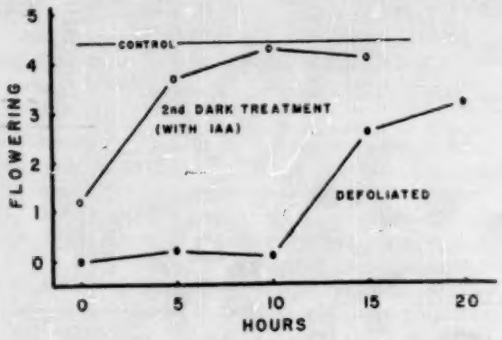


FIG. 1. The stability of the stimulus to a second dark treatment compared to the time of export of the stimulus from the leaf. Following a 12-hour inductive dark period the plants were placed under fluorescent lights (1000 fc) for the specified times then given either a second dark treatment (5 hrs darkness with IAA treatment of 50 mg/l) or defoliated as indicated.

time each treated group of plants was removed from the second dark period. These controls remained vegetative, demonstrating that the stimulus was still in the leaves after the second dark treatment.

Figure 1 shows that if the second dark treatment is given immediately following the inductive dark period, nearly complete inhibition is observed. If the second dark treatment is delayed its effectiveness is reduced until, after exposure of the plants to 6 to 8 hours of light, the flowering response can no longer be affected by the second dark period. Since the stimulus was still in the leaves but was no longer destroyed by the second dark treatment, some change must have taken place in the nature of the stimulus or the leaf during the time it was exposed to the light making the second dark treatment no longer effective. This change will be referred to as the stabilization of the stimulus.

EXPERIMENT 2: In an attempt to define more clearly the nature of the destruction of the stimulus, the effect of temperature on the second dark period was examined. The plants were given the usual 12-hour inductive dark period, terminated by 10 minutes of light. Some of the plants were then treated with IAA, treated and untreated groups were placed in the usual dark room (20–25° C) and similar groups in a refrigerated dark room (3–5° C). After 5 hours all groups were returned to the fluorescent lights.

It may be seen (table I) that at low temperatures the effectiveness of the second dark period is markedly reduced as compared to the usual temperatures used, even with IAA application. These results are interpreted to mean that the second dark treatment results in the destruction of the stimulus by a thermochemical reaction.

EXPERIMENT 3: In a number of experiments, it was desirable to determine the time of movement of the stimulus from the leaves. This was accomplished by removing all the leaves of a size greater than 0.5 cm in length from groups of plants at specified times after the end of the inductive dark period. In order to insure that the small leaves often present on the lateral buds would not contribute stimulus, the lateral buds in the axils of all mature leaves were removed at the same time. The plants were kept under the fluorescent lights from the end of the dark period until after defoliation and later returned to the long day greenhouse.

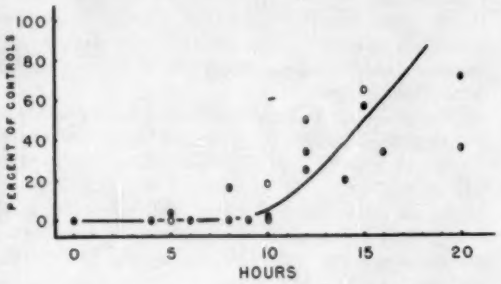


FIG. 2. The time of movement of the flowering stimulus out of Xanthium leaves following a 12-hour inductive dark period. The plants were placed under fluorescent lights (1000–1200 fc) following the dark period and groups of plants were defoliated at various intervals thereafter. The results are expressed as percentage of the average stage of flowering compared to the controls. The results presented here are from seven separate experiments.

In certain experiments defoliation of some plants was delayed 7 days following the dark period and it was found that, in these cases, the flowering response was fully equal to that of the undefoliated controls. Since there is little morphological change in the growing point before such defoliations, it may be concluded that a continuous supply of metabolites from mature leaves is not a necessary factor during the 3-week period allowed for development of the floral primordia. It also supports the contention that injury which would interfere with floral initiation and development is not caused by the defoliation procedure.

The results of 7 separate experiments are presented in figure 2, demonstrating that the stimulus ordinarily does not begin to move out of the leaves

TABLE I

INFLUENCE OF TEMPERATURE ON THE SECOND DARK PERIOD EFFECT *

TEMP. °C	AVERAGE STAGE OF FLOWERING	
	NO IAA	IAA (50 MG/L)
20–25	3.0	1.0
3–5	3.9	3.1

Control: Average stage of flowering—4.4

* Following a 12-hr inductive dark period (terminated by light), the plants were given a 5-hr second dark period under the conditions indicated.

TABLE II

THE EFFECT OF TEMPERATURE ON THE PHOTOCHEMICAL STABILIZATION OF THE FLORAL STIMULUS *

TEMP. °C	HRS OF LIGHT						
	0	0.5	1	2	4	5	8
30	32	32	50	36	64	..	81
5	..	21	50	29	69	..	**
Control: Average stage of flowering—4.2							
30	27	..	..	..	..	88	..
5	..	..	..	..	..	92	..
Control: Average stage of flowering—4.9							
30	0	..	22	..	59	..	..
5	..	..	15	..	73	..	..
Control: Average stage of flowering—4.1							

* The results are expressed as a percentage of the average stage of flowering of the control which received no second dark treatment. Treated plants were given a 12-hr inductive dark period, treated with light (1200 fc) at the temperature indicated, then given a second dark treatment (5 hrs darkness with IAA). The results of 3 separate experiments are presented.

** Plants badly wilted.

until approximately 10 hours after the termination of a 12-hour dark period. A level of flowering approaching that of the controls may be obtained if the leaves are allowed to remain on the plant for about 24 hours after the end of the inductive dark period.

EXPERIMENT 4: In further experiments the exposure to light of high intensity was given some plants at a low temperature (4 to 6° C) and compared to the effectiveness of the stabilization process at normal temperatures (ca. 29° C). After an inductive dark period, plants were placed at the desired temperatures under fluorescent lights giving intensities at the leaf surface of approximately 1200 fc. After various time intervals groups of plants were removed and the remaining non-stabilized stimulus was destroyed by exposure to a second dark treatment (IAA and 5 hours darkness at about 25° C). They were then returned to fluorescent lights at normal temperatures.

These experiments (table II) seem to indicate that the stabilization process progresses equally rapidly at these low temperatures, giving a Q_{10} of approximately one for temperatures between 5° and 30° C for the stabilization process. This would indicate that the rate-limiting reaction in this process is not a thermochemical reaction.

EXPERIMENT 5: It has been shown above that the presumed stimulus is stabilized in light even at low temperatures, while the second dark treatment was ineffective in destroying the stimulus under low temperature conditions. This response makes it possible to determine whether light is necessary to stabilize the stimulus or whether the light prevents its destruction while the stabilization process itself proceeds independently of light (e.g., a diffusion process). If the second possibility is the correct one, then 5 hours of darkness at low temperature would allow stabilization of the stimulus and the stimulus would be unaffected by a subsequent dark treatment at an elevated temperature. If, on the other hand, the light is directly required to stabilize the stimulus (a photochemical reaction), then a dark treatment at high temperature following the low temperature dark period should result in a destruction of the stimulus. The destruction observed should be essentially equal to that obtained when the second dark treatment is given immediately following the inductive dark period.

An experiment to test these possibilities was designed as shown in figure 3, and the results are tabulated in table III. The results show that, after 5 hours darkness or low intensity light (50 to 100 fc), the stimulus is still completely destroyed by a dark treatment at the elevated temperature (3 hours at 40° C). If the plants instead were exposed to 5 hours of high intensity light (1000 fc), at either low or normal temperatures, the stabilization was essentially complete at the end of such treatment. In this experiment the 5 hours of darkness at normal temperature (treatment V) failed to give destruction of the stimulus. This demonstrates the variability in

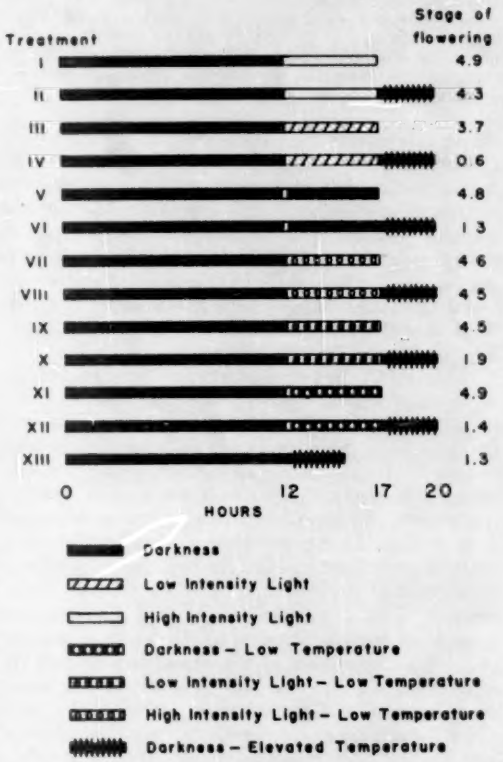


FIG. 3. The effect on the stabilization of the flowering stimulus of various light and temperature treatments for 5 hours immediately following induction. The non-stabilized stimulus was destroyed by exposure to a 3-hour dark treatment at 40° C. See also table III.

Table III

The Effect on the Stabilization of the Flowering Stimulus of Various Light and Temperature Treatments for 5 Hours Immediately Following the Inductive Dark Period *

Treatment during 5-hr stabilization period		% Stabilization **
Temp. °C	Light (fc)	
25–30	1000	83
	100	−13
	0	0
4–6	1000	98
	100	22
	0	2

* The non-stabilization stimulus was destroyed (73 %) by exposure to a dark treatment at elevated temperature (3 hrs at 40° C). See also figure 3.

** Percent stabilization: $\frac{A - (A - B) \times \frac{4.9 - 1.3}{4.9}}{A} \times 100$

where A is the average stage of flowering observed as a result of the experimental treatment without a second dark treatment (see fig 3), and B is the average stage of flowering observed as a result of the experimental treatment followed by the second dark treatment (40° C for 3 hrs). The figures 4.9 and 1.3 are the average stage of flowering observed respectively in the control and in those plants where the second dark treatment immediately followed the inductive dark period.

the effectiveness of the second dark treatment at room temperature, as discussed previously (3). In this treatment as well, subsequent exposure to elevated temperature (treatment VI) demonstrated that the stimulus had not been stabilized in the dark, even though no destruction had occurred. Thus, the conclusion that light is directly necessary for the stabilization of the stimulus is reached independently of the validity of the experiments on the temperature coefficient of the stabilization process.

Included in the above experiment were treatments under conditions of relatively low intensity light, obtained by placing sets of plants at some distance from the fluorescent lights used to provide the light of high intensity. It has been pointed out previously (3), that low intensity light and darkness exert qualitatively the same effect when they follow an inductive dark period. In this experiment, darkness following the inductive dark period resulted in no apparent destruction of the stimulus. The low intensity light, however, gave a marked reduction of the flowering response at normal temperature (treatments III and IV). The destruction of the stimulus precursor by exposure to low intensity light accounts for the negative percentage of stabilization in treatment IV. Few direct comparisons may be made, but low intensity light has not been found previously to be more effective than darkness in causing inhibition.

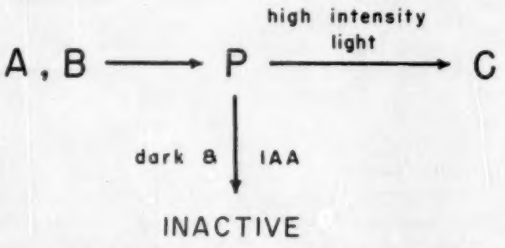
Control plants placed, following induction, under normal temperature fluorescent lights and defoliated 8 hours after the end of the dark period were all vegetative.

Discussion

The length of the critical period of Xanthium is independent of the amount of leaf tissue present (5), and of the number of cycles of treatment (4). This demonstrates that two distinct processes, necessary for the formation of the floral stimulus, are taking place during the dark period. The first may be referred to as the "timing mechanism" which will reach a critical level after a standard length of time, determining the critical period. This process could not represent the total effect of the dark period since if this were the case the effect of darkness on flowering would be quantitative. In order to explain a critical period with the characteristics indicated above it must be postulated that a second process, resulting in the accumulation of the stimulus, will begin upon the attainment of a critical level of the first. In Xanthium this second process usually attains a maximum after approximately 15 hours of darkness, as indicated by the fact that a dark period of approximately this length will usually give maximum flowering. It seems likely that the optimum length of the dark period is governed by the amount of substrate available from the previous high intensity light treatment, shown by Hamner (1) to be required immediately prior to the inductive dark period. Thus at the end of an inductive dark period some condition has been attained which, upon the return of the plant to light, will result in the formation of the floral stimulus in the leaf.

The results presented here indicate that a further reaction, requiring light, normally takes place in the leaf subsequent to the inductive dark period and before the stimulus is translocated from the leaf. Prior to this photochemical reaction the stimulus produced in the inductive dark period may be considered to be present in the form of a precursor, which is changed as a result of the action of light to the final form in which it is exported from the leaves.

These observations may be schematized in the following form in which the A and B represent the



initial high light requirement and the long (inductive) dark period requirement respectively, as defined by Hamner (1). Retaining Hamner's definition of C as the final stimulus transported from the leaf, it has been shown here that an intermediate condition P must be included. The P is the precursor of the final stimulus, present at the end of an inductive dark period which, in the presence of high intensity light,

will give rise to the stable flowering stimulus, but which may be destroyed by a second exposure to darkness, especially in the presence of IAA.

The reaction P → C represents the stabilization of the precursor present at the end of the inductive dark period. It has been shown that this reaction requires relatively high intensity light for rapid (6 hours) completion, although as has previously been pointed out, the light is not an absolute requirement. If the inductive dark period is not interrupted and the plants kept in continuing darkness for 3 weeks they will initiate flowers at approximately the same rate as if they had received a single photoinductive cycle (6). In this case the inductive dark period is not broken and an interruption of induction seems to be necessary before the second dark period is effective in destroying the precursor P.

An alternative explanation would be that, at the end of an inductive dark period, the leaf is capable of destroying the stimulus in the absence of high intensity light. Then, as a result of exposure to light for several hours, the leaf is rendered incapable of destroying the floral stimulus. The P would represent the floral stimulus under conditions in which destruction was possible. The conversion to C would be a change in the leaf to a condition in which destruction of the stimulus would no longer occur under conditions of darkness and IAA treatment.

The overall stabilization process has been shown to have a temperature coefficient approaching unity. This would seem to indicate that a photochemical reaction, requiring high intensity light, was the rate limiting step in the stabilization process. The rate of stabilization has only been adequately established at 5° and 30° C, and studies at intermediate temperatures would be required to definitely establish the effect of temperature on the rate of the reaction. A single experiment, in which an intermediate temperature (16–18° C) was included, supported a temperature coefficient approaching unity. However, as indicated above, the conclusions reached in this paper do not depend on the validity of the temperature coefficient of the stabilization process.

The reaction P → inactive, the reduction of the flowering response as a result of exposure to a second dark period, has been reported previously (3), and the increased effectiveness of the second dark treatment at high temperatures will be reported in greater detail at a later date. It is possible that the inactivation of the precursor might represent a reversal of the reaction A, B → P. Since it is not as yet known whether this is the case, it is necessary to separate the two processes until their identity may be established. Whether the second dark period and IAA act on the same reaction is also uncertain, although the demonstration of an apparent interaction (3) makes it appear likely that this is the case.

It should be emphasized that the partial processes indicated here do not necessarily represent single chemical or physical reactions but may include a series of reactions. The partial processes would then represent only those steps in the overall process affected by the particular environmental conditions to which the plants have been exposed. The separation of the various partial processes as reported here should help make possible more discriminating experiments designed to elucidate the various chemical steps involved in the formation and transport of the flowering stimulus.

Summary

1. The flowering response of Xanthium may be markedly reduced by treatment with IAA and a second dark period following a 12-hour inductive dark treatment.

2. Several hours of high intensity light following the inductive dark period renders the inhibitory dark treatment no longer effective. This is referred to as the stabilization of the floral stimulus.

3. It is postulated that a precursor of the floral stimulus is present at the end of a 12-hour inductive dark period which may be destroyed by IAA and darkness or converted to the floral stimulus by high intensity light.

The authors wish to express their appreciation to the Northern Illinois Natural History Society for collecting the burs used in this study.

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SOME EFFECTS OF ETHANOL EXTRACTS OF POTATOES ON THE ACTIVITY OF A PHOSPHORYLASE PREPARATION[1]

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Arreguin-Lozano and Bonner (2) have reported that when potato phosphorylase, prepared by the original method of Hanes (5), acts upon glucose-1-phosphate (G-1-P) in the presence of material extracted from potatoes by 80 % ethanol, the course of the reaction may be considerably modified. It was claimed that addition of extracts from potatoes stored at 25° C reduced the color intensity of iodine complexes of the polysaccharides formed, whereas no effect was observed when extracts from potatoes stored at 0° C were added. Color intensity was measured in the Klett-Summerson colorimeter, presumably using a red filter, and it was assumed that the readings provided a valid measure of the relative phosphorylase activity of the enzyme digests under all the experimental conditions used. Accordingly it was concluded that an inhibitor of phosphorylase was present in potatoes stored at 25° C which disappeared after two to three weeks storage at 0° C. It was further suggested that the supposed inhibitor is an important factor in regulating phosphorylase action and that its absence at low temperatures accounts for the well known sweetening phenomenon. Potato phosphorylase has proved difficult to prepare free from contaminating enzymes which can act upon the polysaccharides derived from G-1-P, and Hanes showed that secondary degradation of the reaction products occurred with preparations made by the original procedure. For this reason measurement of the intensity of iodine stains alone is unlikely to provide an accurate measure of the relative phosphorylase activity of such preparations. The effects of ethanol extracts have now been re-examined, following the course of reaction by changes in the color intensity of iodine stains, by measuring the free phosphate released and as well by a study of the reaction products. The phosphatase (or a system yielding free glucose and phosphate from G-1-P) contaminant of the type of enzyme preparation used has been shown (10) to account for only a very small fraction of the free phosphate produced, so that estimates of free phosphate are likely to provide a more trustworthy guide to phosphorylase activity than are measurements of iodine stains.

The observation that addition of ethanol extracts of potatoes stored at 25° C profoundly affects the iodine staining properties of the reaction products has been confirmed, but concurrently with the reduction in intensity of iodine stain there was an increase in the amount of phosphate released, suggesting that phosphorylase action was accelerated and not inhibited. It is considered that these effects of ethanol extracts can be attributed to two major factors, the presence of a naturally occurring priming material in the extracts and of a factor modifying the nature of the polysaccharide derived from G-1-P. Experiments concerning these factors are described in the present communication.

No significant difference was observed between the action of extracts from potatoes stored at 25° C and 0° C, although a large increase in sugar content occurred during the cold storage period. The results thus not only do not support the view that there is a specific inhibitor of phosphorylase in potatoes whose activity is controlled by storage temperature, but indeed suggest that phosphorylase is activated by the priming material present in all such extracts. It is recognized that an explanation of the failure to record a differential effect of temperature is not yet forthcoming. In the light of the present work the way in which alteration of the constituents of the ethanol extracts due to storage temperature might affect the course of the reactions studied would seem to depend on a number of complex interactions and much additional data is required before a definitive conclusion can be reached.

MATERIALS AND METHODS

PREPARATION: Potatoes bought in the market were stored at 25° C and 0° C for two or three weeks. They were then cut up and lots of 100 gm macerated in a blendor after addition to 40 ml of water. The volume of the macerate was measured and ethanol was added to make a final concentration of 80 %. The material was then extracted by refluxing for two hours. The ethanol extract was filtered when cool, the ethanol removed in vacuo, and the resulting aqueous liquid again filtered. The precipitate was washed and the filtrate made up to 50 ml. In some experiments the 100-gm lots were dropped at once into boiling ethanol and heated for 30 mins. The mixture was then macerated, the ethanol concentration made to 80 % and the liquid then treated as described above. In both procedures the final aqueous liquid contained the material extracted by ethanol from 2 gm fresh weight of potato per ml. This liquid is termed E–E.

Potato phosphorylase was prepared by the method of Hanes (5), the ammonium sulfate precipitations being made at pH 7.0. The final precipitate was taken up in 0.1 M citrate at pH 6.7 and the solution freeze dried. As required, solutions containing 12 mg/ml were prepared and used as enzyme. Such preparations contain a number of contaminants which may act upon the polysaccharide derived from G-1-P. The term "phosphorylase" action is therefore used here to denote the overall behavior of this preparation.

Glucose-1-phosphate was the preparation of Porter (9). Phosphate was estimated by the method of

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Fiske and Subbarow (4). From the digests samples of 0.1 ml were withdrawn and treated as already described (10).

Absorption by the iodine complexes of the polysaccharides was measured in the Eel colorimeter using a red filter (Ilford, 608) with transmission between 650 and 700 mμ. The colorimeter readings are a measure of the relative intensity of blue color and are here termed absorption values (AV). The iodine complexes were prepared as already described (9), but using 0.1 ml samples from the digests.

Separation of the Reaction Products: To 0.8 ml of absolute ethanol was added 0.2 ml of the digest and the mixture allowed to stand for 24 hours. The precipitated salts and long chain polymers were removed by centrifugation. The supernatant liquid was evaporated to dryness in vacuo, the residue taken up in 0.1 ml of water and transferred to paper for chromatographic separation. Development was effected with propanol-ethanol-water mixtures (1) and the papers sprayed with benzidine to detect the saccharides (3).

Enzyme digests were held at 30° C and at pH 7.0, and usually had a total volume of 2 ml. They contained 0.6 ml of 0.125 M G-1-P, 0.25 ml of 0.2 M maleate buffer pH 6.5, 0.2 ml of 0.2 % soluble starch, 0.6 ml of phosphorylase and 0.35 ml of E–E, other additions or water. These conditions approximate to those quoted by Arreguin-Lozano and Bonner except that the initial concentration of G-1-P was 0.037 M, instead of 20 mg of "Cori ester" in 10 ml (0.0055 M, calculated assuming that "Cori ester" is the dipotassium salt of G-1-P crystallized with two molecules of water). The amount of buffer added was not sufficient to overcome the buffering capacity of the enzyme preparation and the measured pH of the digests was 7.0. In some experiments maltopentaose was used to prime the reaction instead of starch.

Experimental Results

The effect of adding material extracted by 80 % alcohol (E–E) from potatoes stored at 25° C on the course of "phosphorylase" action is illustrated in figure 1. The primer concentration, 20 mg per 100 ml of starch is suboptimal, so that the reaction did not proceed at maximum velocity. There was a small stimulation of phosphate release by addition of E–E, indicating a priming effect (fig 1, curves 1a, 2a). The presence of priming material was confirmed by adding E–E to digests from which starch was omitted, when three times as much phosphate was released in 40 min as in a parallel unprimed digest. The course of this reaction is shown in insert to figure 1. The very large effect of adding E–E upon the intensity of blue iodine stain of the polysaccharide formed (AV) is shown in figure 1 (curves 1b, 2b). The maximum AV reached in the presence of E–E was less than a third of that attained in its absence. The maximum was reached earlier and subsequently the absorption values fell steadily with time In the presence of E–E the color was never blue to the eye, the samples exhibited a variety of brown, reddish brown and orange stains usually associated with mixtures of dextrins. The possibility that Q enzyme might be present has been considered, but the evidence to be put forward indicates that the color of the iodine stains cannot be accounted for by formation of branched polysaccharides. Observations such as these cannot indicate the amount of polysaccharide produced, but they do suggest that the presence of E–E may have prevented formation of long chain linear polymers which stain iodine blue while simultaneously increasing the amount of G-1-P converted.

Finally the absolute fall in AV from a low maximum after 45 min reaction time (curve 2b), when the rate of phosphate release is only beginning to fall off (curve 2a), shows clearly that the average chain length of the reaction products is somewhat rapidly reduced when E–E is present.

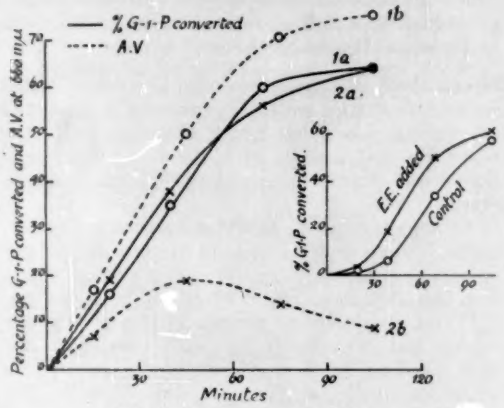

Fig. 1. Course of "phosphorylase" action as affected by addition of material extracted from potatoes by ethanol (E–E). Digests. 2 ml at 30° C and pH 7.0 containing 0.037 M G-1-P, 0.02 M maleic buffer at pH 6.5 (see text), 0.6 ml potato phosphorylase, 0.2 mg soluble starch, and 0.35 ml E–E or water. Curves. 1a and b. Controls. 2a and b. E–E added. Insert. G-1-P converted in the absence of starch primer.

The effect of adding E–E from potatoes which had been stored at 25° C and at 0° C is shown in figure 2. Both extracts led to a small increase in the amount of phosphate released, as indicated by the symbols above the curve showing the course of phosphate release in the absence of E–E. Both extracts also led to large reductions in the AV's, but there was only a very small difference due to the storage temperature of the potatoes. The products of reaction after 3 hours, were, in the absence of E–E, mainly blue iodine staining amylose, together with small amounts of maltotriose, maltose and glucose. On the basis of previous experience (10), these latter substances are considered to arise from the action of α amylase and phosphatase contaminants of the enzyme preparation. In the presence of E–E there were large amounts of glucose, fructose and sucrose in the digests derived from the

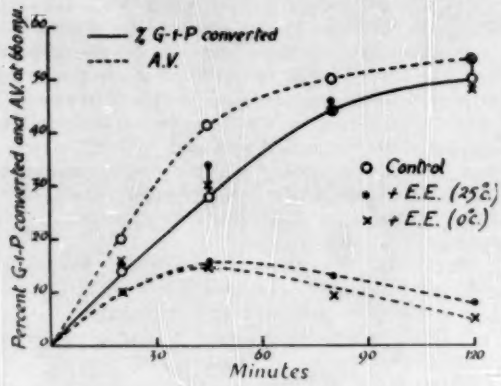

FIG. 2. Course of "phosphorylase" action as affected by addition of E–E from potatoes stored at 25 or 0° C for 3 weeks. Digests. As in figure 1.

extract itself, a little maltose and a series of higher saccharides starting with maltotriose and extending to the starting line in the paper chromatogram. The conclusion that addition of E–E led to formation of short chain dextrins instead of amylose was thus confirmed.

Chromatograms of highly concentrated E–E revealed faint spots in the areas in which maltopentaose or hexaose might be expected to occur. Accordingly E–E from 200 gm of potato which had been stored at 0° C was evaporated to dryness in vacuo. The dry residue was exhaustively extracted with ether and then taken up in 25 ml of water. Of this solution 0.5 ml was applied to paper in a series of spots. The developed chromatogram was cut into two sections, one containing the possible maltodextrins and the other the sugars. These sections were eluted with water, the extracts evaporated and added to "phosphorylase" digests as before. The effect of adding the eluate containing the supposed maltodextrins to unprimed reaction mixtures as compared with that of adding E–E is shown in figure 3. Both extracts caused large and similar increases in the amount of phosphate released (fig 3, curves 1a, 2a, 3a), but the effects upon the AV's were quite different. Addition of the complete E–E led to a small initial increase in AV, due to acceleration of the phosphorylase reaction, but the increase was soon arrested and after 75 min the AV's reached a very low maximum (fig 3, curves 1b, 2b). The iodine stains of the polysaccharides at the maximum AV were red-brown. Addition of the fraction of E–E containing the priming material only, led to a much greater initial increase in the AV's and there was no subsequent arrest of this increase (fig 3, curve 3b). After two hours the polysaccharide formed stained iodine a brilliant blue. Dividing the chromatogram into two sections had thus separated the factors responsible respectively for the increase in phosphate release, and the production of short chain polymers. The latter factor was located in the section containing the sugars.

A syrup was prepared containing the priming material by extracting 1 kg of potatoes with ethanol and passing the concentrated extract through a charcoal column, eluting first with water and then with dilute ethanol (12). After removal of any maltotriose with 15 % ethanol, the remaining material was eluted with 30 % ethanol. A considerable amount of tyrosine separated from this eluate and was removed. The eluate was then evaporated to a syrup and shown to prime the phosphorylase reaction. It was found by paper chromatography to contain dextrins which corresponded in location on the paper to maltotetraose and higher polymers. When β-amylase, prepared by the method of Hopkins, Murray and Lockwood (6), was allowed to act on this material the products were maltotriose, maltose and a little glucose. It was therefore concluded that the syrup was a mixture of α-1:4 linked maltodextrins. The possibility was considered that these dextrins might arise enzymatically during maceration of the potatoes. However, when potato slices were cut, dropped at once into boiling ethanol and subsequently macerated, the final extracts still contained material priming the phosphorylase reaction.

In figure 4 is shown the effect of adding the eluate from the section of the paper containing the sugars (see above) to "phosphorylase" digests, in the presence of maltopentaose as primer. There was little or no effect upon phosphate release (fig 4, curves 1a, 2a), but a very marked effect upon the AV's (fig 4, curves 1b, 2b). The maximum AV reached was somewhat higher than in the experiments where complete E–E was added (see figs 1 and 2). This higher value may in part be due to the effects of a higher primer concentration.

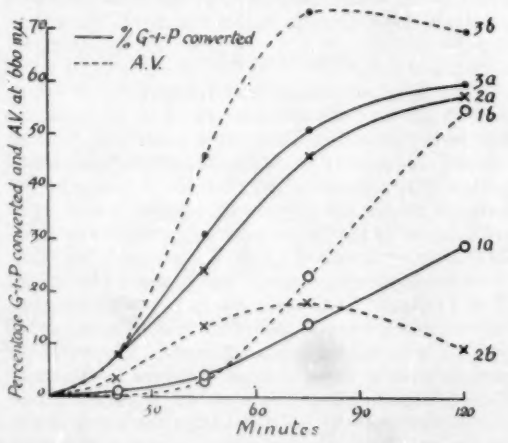

FIG. 3. Course of "phosphorylase" action as affected by addition of E–E, or the fraction of E–E containing primer. (See text.) Digests. As in figure 1 except starch omitted. Curves. 1a and b. Control. 2a and b. E–E added. 3a and b. Fraction of E–E containing primer added.

Further examination of the effects upon the "phosphorylase" reaction of the sugar containing fraction of E–E was made by dividing a developed chromatogram into ten sections. For this purpose Whatman No. 3 MM paper was used and a 2-inch strip containing the constituents of 0.7 ml of the original E–E was cut up. The top section contained the maltodextrin area, and the nine lower sections the several sugars and large amounts of amino acids. The paper cuts were shaken with 10 ml of water, the water drawn off through a filter stick and extraction repeated. The extracts were evaporated, taken up in 0.7 ml of water and "phosphorylase" digests prepared containing 0.35 ml of each fraction. Maltopentaose was used in optimal amounts to eliminate differential effects of primer. The course of reactions was followed by measuring AV's. A family of curves was

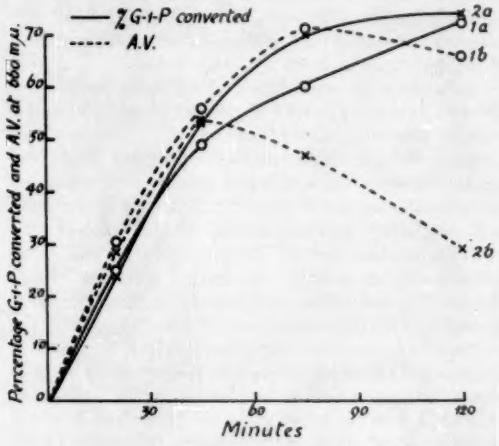

FIG. 4. Course of "phosphorylase" action as affected by addition of the fraction of E–E containing sugars. (See text.) Digests. As in figure 1 except for addition of 0.2 mg maltopentaose instead of starch. Curves. 1a and b. Controls. 2a and b. Sugar containing fraction of E–E added.

obtained, similar to those of figures 1 and 2 illustrating the course of reaction in the presence of E–E, but in which the maximal values varied considerably. When the maxima were low, that is the effect was greatest, they occurred after about 45 minutes, but when they were high after 75 minutes. The maximum AV's for each of the ten extracts is shown in figure 5, together with a record of the sugars present in each digest. Owing to the rather large amounts of sugar present separation was not quite complete, and it was also observed that sucrose was slowly inverted by the "phosphorylase" preparation. Nevertheless it is at once apparent that there is little effect upon the maximum AV values in the absence of sugar containing fractions, and that the greatest effect in reducing these values occurs in the digests in which glucose was present. Accordingly the effect was examined of adding glucose in the highest concentration estimated

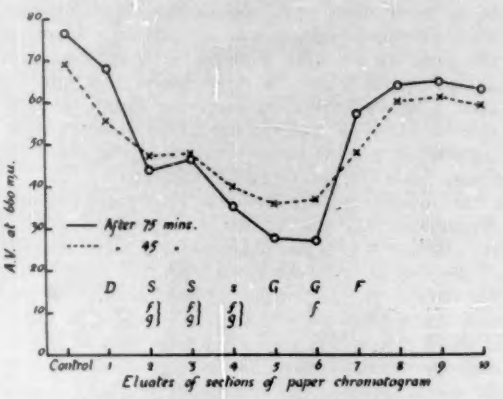

FIG. 5. Effect of addition of concentrates of ten fractions of E–E (see text) on the maximum AV attained during the course of "phosphorylase" action. Maxima were reached after 45 or 75 minutes. Digests. 1 ml at pH 7.0 and 30° C, containing 0.032 M G-1-P, 0.15 ml 0.02 M maleic buffer, 0.25 mg maltopentaose, 0.25 ml potato phosphorylase. Sugars present. D maltodextrins, S much sucrose, F and G much fructose and glucose. s, f, g small amounts of sugar.

to be present in the E–E fractions, namely 3.8 mg per digest, with the result shown in figure 6. Comparison of figure 6 with figure 4 indicates that the effect of glucose addition was remarkably similar to that of the fraction of E–E containing sugars, namely little or no effect upon phosphate release and a large effect upon the AV's. Similar experiments adding fructose or sucrose did not exhibit any effect upon AV's. Chromatograms of samples of the digest to which glucose had been added showed the presence of maltodextrins. The marked action of glucose in limiting the length of the polysaccharide chains produced clearly could not

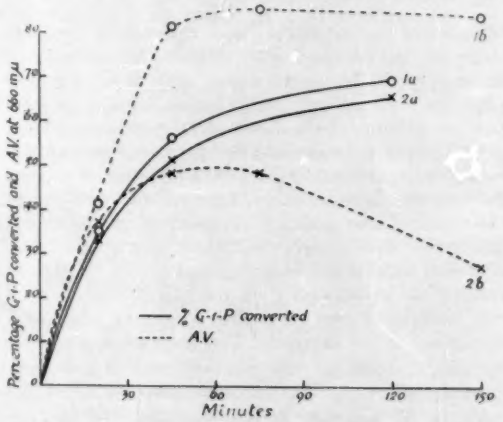

FIG. 6. Course of "phosphorylase" action as affected by the addition of glucose. Digests. As in figure 1 except for addition of 3.8 mg glucose instead of E–E. Curves. 1a and b. Control. 2a and b. Glucose added.

be a direct effect upon the phosphorylase reaction, since phosphate release was not altered. Moreover the presence of large amounts of maltotriose and maltopentaose among the final products of reaction after 3 hours incubation suggested that a transfer reaction might be involved due to the presence of the transferring enzyme recently reported to be present in potato juice (8) and termed D enzyme.

Qualitative tests showed that the "phosphorylase" preparation was able to act upon maltopentaose in the absence of free phosphate yielding small amounts of dextrins of sufficient chain length to stain iodine red brown. In the presence of large amounts of glucose the reverse reaction may be expected to occur, in which glucose accepts maltodextrin residues and by disproportionation lowers the average chain length of the maltodextrins. The experiment of adding glucose to a "phosphorylase" digest was therefore repeated using glucose uniformly labeled with C^{14}. The course of reaction was similar to that depicted in figure 6. Reaction was stopped after 3 hours by addition of ethanol to 80% concentration. At this stage the products retained only slight iodine staining properties. Separation of the digest constituents was carried out on paper as before and the paper cut into appropriate strips at right angles to the direction of solvent flow, on the basis of guide strips treated with benzidine and of the position of areas of radioactivity. The strips were eluted with water, the eluate being delivered onto metal counting plates (7). After recording the radioactivity on the plates, the material was washed off and estimated as glucose after acid hydrolysis of the dextrins. The results are presented in table I. All of the maltodextrins found were labeled and of the total counts recovered from the paper 41% were in the maltodextrins and maltose and 59% remained in the added glucose. Short chain maltodextrins had thus been formed by D enzyme action, with glucose as the acceptor molecule.

TABLE I

RADIOACTIVITY OF SACCHARIDES RESULTING FROM "PHOSPHORYLASE" ACTION IN THE PRESENCE OF GLUCOSE UNIFORMLY LABELED WITH C^{14} *

	AMOUNT ELUTED MG × 10	CPM **	% TOTAL CTS.	CPM/MG $\times 10^{-3}$
Glucose	3.47	15,743	59	45
Maltose	0.75	962	4	13
Maltotriose	3.34	4,972	18	15
Maltotetraose	3.18	2,910	11	9
Maltopentaose	2.02	1,440	5	7
Maltodextrins with over 5 hexose residues	1.22	758	3	5
Totals	13.97	26,788		

* Reaction mixture as for figure 6. Products separated by paper chromatography, eluted from paper, and estimated as glucose after hydrolysis. See text.
** Counts per minute.

DISCUSSION

The presence in potato juice of a number of enzymes which can act upon glucose polymers may result in reaction products other than synthetic amylose when juices or crude phosphorylase preparations are allowed to act on G-1-P. The amounts of such products, either glucose polymers of much shorter chain length than can be included in the term amylose, or branched polysaccharides if Q enzyme is active, will vary with the relative amounts of the several enzymes present. Average chain length may also be affected by the number of non-reducing chain ends available to accept glucose residues, and this number may vary during the course of the reaction, as for example by amylolysis. Provided the transfer of glucose from G-1-P, that is phosphorylase activity, is independent or nearly so of concurrent changes in glucose polymers brought about by other enzymes, similar phosphorylase activity may be associated with reaction products varying from amylose to short chain dextrins which do not stain iodine.

In seeking an explanation of the very remarkable effect of ethanol extracts of potatoes upon the iodine staining properties of the products of "phosphorylase" action it became apparent that the major effect was indeed not upon the number of glucose residues transferred but upon the average chain length of the reaction products. Re-examination of the "phosphorylase" preparation led to the detection of two more contaminants, namely D enzyme and invertase. Since free glucose can act as an acceptor in the D enzyme system (8, 11) attention was at once drawn to the possible importance of the glucose which is added in the ethanol extracts. From the results of the experiments reported here it is now suggested that the greater part of the effect upon the chain length is due to this addition of glucose which makes possible simultaneous action of phosphorylase and D enzyme. At the outset of the reaction, when G-1-P is the substrate, polysaccharide synthesis proceeds until dextrins of sufficient chain length are present to initiate D enzyme action. Disproportionation then begins by transfer to the added glucose, leading to the formation of maltodextrins staining iodine brown or red (11). Formation of chains long enough to stain iodine blue is prevented, and when the rate of formation of new α-1:4 links diminishes as phosphorylase equilibrium is reached the average chain length of the reaction products begins to fall (figs 1 and 2). The final products are mainly maltohexaose, pentaose, tetraose and triose and the solution does not stain iodine. The results of table I suggest that one glucose residue is incorporated in each maltodextrin molecule. The amount of C^{14} involved, 41% of the total added, requires the breaking of about 1 in 7 of the links initially formed by phosphorylase. The production of short chains cannot therefore be due primarily to amylolytic degradation of polysaccharide since in this case the dextrins would not be labeled. The presence of a small amount of labeled maltose, which cannot be attributed to D enzyme action (11) may perhaps

be explained by a slow amylolysis of the first products of the transfer reaction.

There appears to be no competition between the phosphorylase and D enzyme with the particular concentrations of reactants used since phosphate release remained unaffected by the glucose addition. The amount of primer present probably exerts some effect upon the maximum chain length reached. The lower AV values found when primer level is suboptimal (cf figs 1 and 4) might simply be due to the decrease in the initial velocity of polysaccharide synthesis, without alteration of D enzyme activity. Other considerations however cannot be excluded, since it was found that if glucose was present in a "phosphorylase" digest, not containing any added primer, so that the initial reaction was very slow, it was without effect on the AV and in time amylose was synthesized. Further information is necessary to elucidate the inter-relations between primer level, glucose concentration, and the maximum average chain length reached.

The E–E from potatoes stored at 0° C had a glucose content of 10 mg/ml as compared with 3 mg/ml in potatoes stored at 25° C so that the absence of any marked difference in the effects of these extracts upon the "phosphorylase" action (fig 2) must suggest that glucose concentration was not limiting at the lower level for the small amount of D enzyme present. No data are yet available as to the effect of glucose concentrations lower than those found in these E–E's on the average chain length of the dextrins formed, and the existence of further unknown factors affecting chain length is not, of course, excluded. It must be admitted that a differential effect of temperature of the type recorded by Arreguin-Lozano and Bonner (2) cannot readily be explained on the hypothesis that glucose is the major factor preventing the formation of polysaccharides staining iodine blue. Presumably glucose contents of potatoes stored at 0° C will always be higher than those of potatoes stored at 25° C. However this may be, there can be little doubt that glucose can exert a profound effect on the nature of the reaction products when "phosphorylase" preparations are contaminated with D enzyme.

The occurrence of maltodextrins in the ethanol extracts, although having only a small effect on the reactions discussed here, is perhaps of some interest, as the presence of such possible intermediates in starch metabolism has not so far as is known been reported in the potato. It is clear that only very small amounts are present and the possibility arises that they are produced enzymically during preparation of the macerates, although it will be remembered that in vitro amylases do not attack the intact starch grain, which would presumably be the possible source of dextrin. However, evidence of the presence of dextrin was obtained when the potato extracts were made, by dropping the cut slices at once into boiling ethanol and only macerating after boiling for half an hour. If the dextrins arise as artefacts, and are not natural constituents of the potato, they must be produced during the actual cutting up of the tubers.

Summary

When potato phosphorylase was prepared by the original method of Hanes, the polysaccharides produced by its action, in the presence of material extracted from potatoes by ethanol, stained iodine red or brown instead of the expected blue. The reaction products were found, by paper chromatography, to be maltodextrins. These dextrins were formed by the action of an enzyme contaminating the phosphorylase, which transferred maltosyl or larger units from the initial products of phosphorylase action upon glucose-1-phosphate to the glucose contained in the potato extract. When glucose, uniformly labeled with C^{14} was added instead of the potato extract a similar effect was obtained and a series of maltodextrins was isolated, all of which were labeled.

Release of phosphate from glucose-1-phosphate was increased in the presence of the potato extract, owing to the presence of small amounts of naturally occurring dextrins which acted as primers. A syrup consisting of a mixture of short chain dextrins has been isolated and the mixture found to prime the phosphorylase reaction and lead to amylose synthesis.

The effect of ethanol extracts of potatoes upon the iodine staining properties of the polysaccharides produced by "phosphorylase" action thus appears to be accounted for in the main by addition of glucose to a phosphorylase preparation contaminated with another transferring enzyme. Operation of additional factors is not excluded.

The results do not support the view put forward by Arreguin-Lozano and Bonner (2) that potatoes contain a phosphorylase inhibitor which disappears if potatoes are stored at 0° C. Apart from the fact that it is now shown that phosphorylase activity was increased by addition of ethanol extracts, no difference was observed between the effects of extracts from potatoes stored at 25° or 0° C. Possible differential effects of storage temperature upon the very complex system under study require further investigation.

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THE EFFECT OF ABNORMALLY PROLONGED ALTERNATING PERIODS OF LIGHT AND DARKNESS UPON THE COMPOSITION OF BRYOPHYLLUM CALYCINUM LEAVES[1]

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In a state of nature, succulent plants such as *Bryophyllum calycinum* respond to the diurnal alternations of light and darkness with a closely correlated rhythmic change in the organic acid and starch content of the leaves. However, when the leaves are exposed to continuous darkness, or, alternatively, to continuous light (9), and the changes in composition which occur are compared with those observed under conditions of normal diurnal alternation of light and darkness, certain differences in the behavior of a number of the components begin to become evident as soon as the period of darkness, or of illumination, exceeds the length of a normal night or of a normal day.

Bennet-Clark (1) some twenty years ago showed that the rapid rise of organic acids that occurs when leaves are first placed in the dark is succeeded by a slow fall which begins after about 12 hours. He also reviewed the early literature on the marked changes in the respiratory quotient which take place at this critical period, an aspect of the problem which has been the theme of much recent research especially by Thomas and his associates (6). Wolf (14) has also examined the changes in the respiratory quotient, as well as in the acidity and the carbohydrate composition, in experiments in which leaves of succulent species have been cultured in darkness for several days. All of these workers have found that the respiratory quotient is close to zero at the start of the dark period but rises to unity in about 12 hours.

In order to obtain additional information on the alterations which occur in the normal course of the chemical reactions on prolonged culture either in light or darkness, samples of *Bryophyllum calycinum* leaves have been exposed to continuous artificial illumination for 24 hours and then to 24 hours of darkness. No intervening period of gradual reduction of the intensity of the light in simulation of twilight was employed since the interest was concentrated upon the response, whether immediate or delayed, to sudden darkness following upon an abnormally long period of illumination.

[1] Received July 19, 1954.

Experimental Procedure

The ten samples of leaflets used were collected by the statistical method (13), starting at 5:55 A.M. (sunrise 5:53 A.M.) on October 7, 1952, from 20 plants that had been transplanted to sand in February. The plants had been grown with use of a culture solution through the summer, all branches and suckers being removed, and were about 4 ft high. Each sample contained 60 leaflets 3 of which had come from each of the 20 plants and each of the 30 leaflet positions (i.e., 5 leaflets from each of the 6 leaves) on an individual plant was represented twice in each sample. Because of occasional variations from plant to plant, such as loss or malformation of an individual leaflet, a few substitutions of other but similar leaflets were necessary as the sampling process was carried out. The technique for handling the samples and references to the analytical methods employed have been given in a previous paper (12). Zero hour was taken as 7:00 A.M. when the control sample was placed in the drying oven.

The culture troughs were set up in an insulated room with controlled temperature under a bank of fluorescent lights which gave an intensity of from 700 to 800 fc at the surface of the leaves. During the light period, the temperature remained essentially constant at 20 to 22° C but, when the lights were turned off after 24 hours, the cooling equipment brought the temperature down to 16° C after 8 hours. The compressor was then turned off and the temperature slowly rose to 20° C. The temperature record is shown in table I.

Samples were removed at the times shown in table I, these being chosen so as to give maximal information on the anticipated changes in rate of the chemi-

TABLE I

FUNDAMENTAL DATA ON SAMPLES OF BRYOPHYLLUM CALYCINUM LEAVES COLLECTED BY THE STATISTICAL METHOD AND SUBJECTED TO CULTURE IN WATER FOR 24 HRS IN LIGHT FOLLOWED BY 24 HRS IN DARKNESS

SAMPLE NO.	CULTURE PERIOD	CONDITIONS OF CULTURE *	PER SAMPLE			PER KG INITIAL FW				
			FW AT START	FW AT END	EQUILIBRATED DRY WT	EQUILIBRATED DRY WT	TOTAL N	PROTEIN N	ASH	ALKALINITY OF ASH
	hr	*°C*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*meq*
1	0	.....	413.28	413.3	50.11	121.3	2.21	1.76	10.4	213.0
2	8	L:20	420.32	413.7	49.83	118.6	2.22	1.73	10.3	211.1
3	12	L:22	420.57	406.5	50.34	119.7	2.25	1.72	10.5	209.5
4	18	L:22	415.45	385.2	50.15	120.7	2.16	1.70	10.6	215.6
5	24	L:22	424.71	385.6	51.85	122.1	2.28	1.72	10.5	217.6
6	28	D:18	414.19	374.7	50.54	122.0	2.24	1.70	10.5	215.1
7	32	D:16	408.83	378.8	50.72	124.1	2.23	1.73	10.8	214.8
8	36	D:17	408.10	377.1	51.32	125.8	2.24	1.72	10.7	215.2
9	41	D:20	421.02	386.2	52.71	125.2	2.29	1.70	10.8	219.6
10	48	D:20	414.03	378.6	50.56	122.1	2.19	1.72	10.4	216.0
Mean			416.05		50.81		2.23	1.72	10.55	214.8
S.D.			5.43		0.89		± 0.04	± 0.02	± 0.16	± 2.9
C.V. (%)			1.3		1.8		1.8	1.2	1.5	1.4

* The symbol L signifies in light; D, in darkness.

cal reactions under study. Table I also shows the initial and final weights of the samples together with evidence on the constancy of their composition. In spite of the slight liberties that were necessarily taken with the rigid application of the statistical method of collection, the initial fresh weights of the samples were exceptionally constant, the coefficient of variation being only 1.3 %. The composition with respect to total nitrogen, protein nitrogen, ash, and alkalinity of ash was also constant within satisfactory limits.

EXPERIMENTAL RESULTS

Inasmuch as the data for organic solids contain the analytical errors in the determinations of both moisture and ash, as well as the sampling error, the curve in figure 1 for this component expressed as gm per kilo of initial fresh weight is somewhat irregular. It shows, however, that there was a slow but apparently continuous uptake of organic material throughout the period of illumination and that this uptake continued for about 12 hours after the lights were turned off. That the organic solids of Bryophyllum leaves may increase for many hours under continuous illumination has been shown in a previous paper (9), and that an increase can also be detected during the first 12 to 16 hours when leaves are exposed to darkness is also known (10). Subsequent loss of organic material after a more protracted period of darkness has been repeatedly observed and is to be expected after the process of acid formation has been completed and losses of organic matter from respiration begin to dominate the situation. The present set of samples accordingly behaved, with respect to the increase or loss of organic solids, in a manner which conformed with previous observations.

The curve for the fresh weight at each point of observation, expressed as a percentage of the initial weight of the same sample, shows an effect of prolonged exposure to light that became apparent as soon as 12 hours had elapsed. Excised leaves exposed to normal diurnal alternations of illumination under similar experimental conditions as a rule lose from 2 to 3 % of their fresh weight during the light period but recover nearly all of this loss at night (5). They at no time within 3 days become notably flaccid. After 12 to 16 hours under continuous light, however, Bryophyllum leaves begin to lose weight and flaccidity soon becomes obvious. This phenomenon was also evident in the present instance. The leaves after 12 hours in light were noted to be slightly flaccid and the leaves in all subsequent samples were recorded as being limp although there was evidence of recovery of some of the fresh weight after the lights had been turned off for 8 hours. It seems clear that significant and apparently only partially reversible damage may be done to the water-holding capacity of the leaves when the illumination exceeds in length a normal daylight period. However, this behavior is not invariably encountered, for, in an unpublished experiment carried out in July 1953, the leaves lost only 3 % during 34 hours of illumination and recovered completely during an ensuing 24-hour dark period. Nevertheless, a second exposure to light brought about excessive loss of fresh weight after 16 hours, and it would therefore appear that this somewhat younger and possibly more vigorous lot of leaves ultimately also suffered damage.

The behavior of the starch (fig 1) followed the anticipated course. Starch increased rapidly for 12 hours and reached a maximum in 18 hours but subsequently remained constant until the lights were turned off. Starch then fell rapidly for 12 hours, but the reactions in which starch was being utilized abruptly slowed down at that point and there was little further loss.

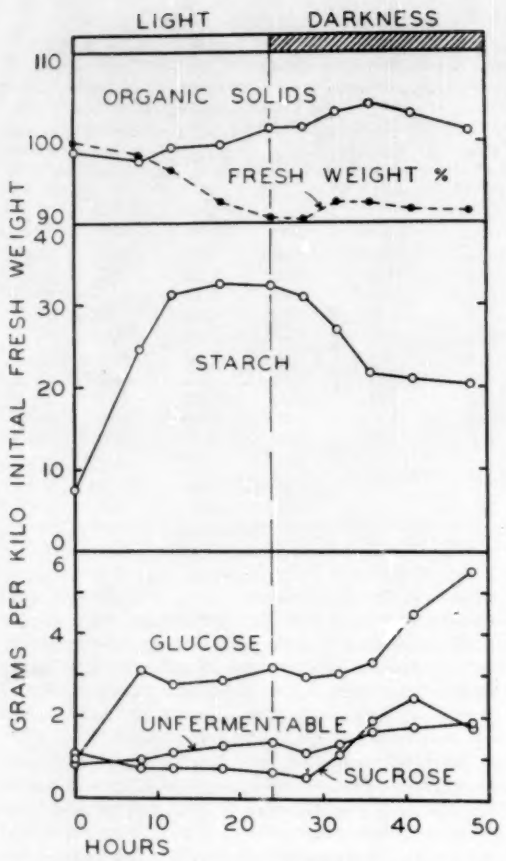

FIG. 1. Changes in organic solids, starch, and soluble reducing carbohydrates in excised leaves of *Bryophyllum calycinum* cultured in water at room temperature for 24 hours in light and 24 hours in darkness.

The sudden change in the rate of utilization of starch after 12 to 16 hours of darkness has been repeatedly encountered (e.g., (3) and unpublished experiments), but different lots of leaves may reach this stage at quite different levels of starch content, the present lot being unusually high in starch when the change occurred. However, the starch metabolism in these leaves was not notably sluggish, as is shown by the large increase during the first 12 hours of exposure to light. It seems possible that excision of the leaves may place a limitation upon the subsequent range of the response of the starch content to changes in light conditions. Indications of such an effect are to be seen in earlier experiments on diurnal variation of starch for 3 and for 2 successive days (5, 10). Alternatively, the failure of the starch to decrease to a low level may have been a delayed result of the prolonged exposure to light.

The behavior of the simple carbohydrates is plotted at the bottom of figure 1 on a scale of ordinates five times larger than that used in plotting the starch. In conformity with previous observations, glucose responded to illumination of the leaves with an immediate increase. However, the increase in 8 hours amounted to only about 2 gm per kilo while that of starch in the same interval was 17 gm. Whether or not the increase in glucose was a result of photosynthesis is not certain. Glucose subsequently remained fairly constant until the critical point after 12 hours of darkness was reached when it again increased.

The so-called unfermentable carbohydrate underwent little change throughout the experiment. There was a slow and nearly continuous rise but it is clear that the moderate drop in temperature in the first few hours of the dark period did not result in an increase in any way comparable to the change in this component that has been observed to occur at 6° C (12).

Sucrose remained constant throughout the light period, as was anticipated, but increased notably

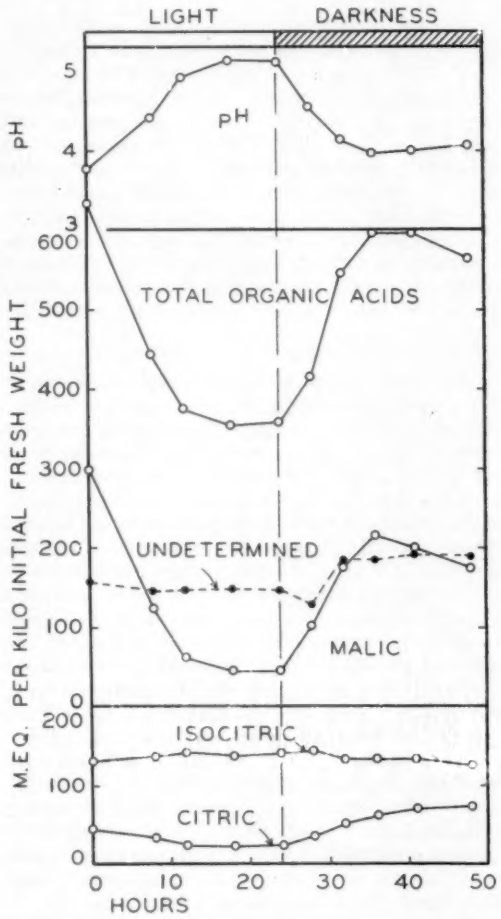

FIG. 2. Changes in organic acids in excised leaves of *Bryophyllum calycinum* cultured in water at room temperature for 24 hours in light and 24 hours in darkness.

shortly after the lights were turned off. It diminished again in the last 7 hours of the experiment. The significance of this behavior is not clear, but the timing of the change was definitely out of phase with the alterations in both starch and glucose.

The behavior of the organic acids is shown in figure 2. The pH increased rapidly for the first 12 hours of illumination and reached a maximum at 18 hours where it remained constant. An immediate drop in pH occurred after the lights were turned off and continued for 12 hours. However, in the last 12 hours of darkness, the pH rose slightly. The explanation of these effects is clearly evident in the curve for total organic acids. Acids were utilized rapidly for the first 12 hours and then slowly for the next 6 hours. There was no further change until after the lights were turned off when a rapid synthesis of organic acids began. This continued for 12 hours but then abruptly ceased and there was a small net loss of acids in the last 7 hours of the dark period. The correlation between the organic acidity and the pH is complete, the coefficient of correlation being -0.973 ($r = 0.872$ for $P = 0.001$ with 8 degrees of freedom).

As is evident from the curves in the lower part of the figure, by far the greater part of the fluctuation in total organic acidity is due to the change in the quantity of malic acid. This dropped rapidly for 12 hours, slowly for another 6 hours, and then remained constant. When the leaves were darkened, malic acid increased rapidly for 12 hours to a maximum and then decreased slowly for the rest of the experimental period. The symmetry of the curve for malic acid with the curve for starch in figure 1 is obvious and the coefficient of correlation between the data for these two components was -0.935 ($r = 0.872$ for $P = 0.001$ with 8 degrees of freedom). An intimate relationship between the reactions which affect the concentrations of these two substances must therefore exist.

Isocitric acid remained essentially constant throughout. The curve suggests a slight increase during the light period and a small decrease towards the end of the dark period, but the data as a whole have a coefficient of variation of 4.1 % which is little if at all in excess of the analytical error. It accordingly seems unlikely that isocitric acid responded significantly to the experimental conditions.

Citric acid behaved as would have been anticipated from previous studies. It decreased during the first 12 hours of the light period to a level which then remained constant, and increased continuously throughout the dark period. Although the changes were not large in terms of actual amount, they were substantial in terms of relative quantities. About one half of the citric acid present at the start had disappeared after 12 hours of illumination; after the lights were turned off, citric acid increased to about three times its minimal level.

The group of undetermined acids remained constant throughout the light period but at first dropped and then increased sharply after the dark period began. Too much significance cannot at present be attached to the apparent changes in these unknown acids since the data are obtained by difference. However, the increase that occurred after 8 hours of darkness was substantial and the higher level was maintained to the end of the experiment. The explanation must await investigation of the detailed composition of this fraction.

Discussion

Consideration of the data plotted in figures 1 and 2, in comparison with the results of studies of the composition of Bryophyllum leaves subjected to normal diurnal alternation of light and darkness (10), or to prolonged illumination (9) or prolonged darkness (11), indicates that the extension of the light or dark period beyond what may be regarded as "normal" has certain well defined effects. The most obvious one with respect to abnormal length of exposure to light is the loss of water-holding capacity of the tissues since this soon becomes apparent owing to the onset of flaccidity. The effect upon the starch and organic acids emphasizes the adaptation of the metabolism of the leaves of this species to the usual length of a daylight period. The normal behavior can be expressed by the statement that malic acid rapidly decreases and starch rapidly increases, these changes being accompanied by a small decrease in citric acid. At the expiration of about 12 hours, however, the rate of change rather suddenly diminishes and the composition of the leaves soon becomes stabilized. Further exposure to light brings about no significant additional alteration in either starch or malic acid content although citric acid may continue to decrease at a slow rate. In effect, the fluctuations in starch and malic acid are rather precisely adjusted in rhythm to the normal diurnal variation in illumination.

There seems to be an upper limit beyond which the starch content of any given lot of Bryophyllum leaves does not increase, although this maximum may differ between fairly wide limits in different lots. Figures close to 30 gm per kilo of initial fresh weight are commonly obtained, but an occasional set of samples may contain as little as 20 gm or as much as 38 gm after exposure to light. There is also a lower limit at which the malic acid content of a given lot of leaves becomes more or less constant, even if illumination is prolonged, and this usually lies between 20 to 40 meq per kilo. Exposure of the leaves to light thus brings them in a period of about 12 hours to a state at which the rates of the chemical reactions in which starch and malic acid are concerned appear to have attained a fairly constant relationship, although there is some evidence that the composition may then vary slightly in response to minor changes in conditions such, for example, as temperature.

Even after the leaves have been exposed to light for an abnormally long period, a sequence of chemical events begins when the lights are turned off which seems to be identical with that observed after nightfall in leaves exposed to the normal diurnal variation of illumination. Starch decreases, malic acid is synthesized with great rapidity, and citric acid increases

slowly. These reactions continue for about 12 hours. At this point, however, a relatively sudden change in the general course of the metabolism supervenes. The rate of decrease of starch becomes slow or stops entirely and, in a few cases observed in this laboratory, a small increase of starch occurred. Malic acid reaches a maximum and then begins slowly to decrease, and if the leaves are kept in darkness for a long time, this substance may even diminish to the level observed at the end of a sunny day.

In a recent paper (12), the behavior of malic acid in leaves cultured in darkness has been attributed to the competition between reactions that bring about its synthesis and reactions that make use of malic acid or of one of its precursors for the production of other metabolic products. These latter reactions appear to be especially sensitive to the temperature at which the experiment is carried out and are greatly depressed in rate if the temperature of the leaves is of the order of 10° C or less. Accordingly, at low temperature, accumulation of malic acid can continue for a considerably longer time than is observed at temperatures of 20° C or upwards. The timing of the sudden apparent alteration of the metabolism of malic acid shown in figure 2 is thus contingent upon the temperature at which the culture experiment is carried out. Nevertheless, even at a temperature as low as 9° C (4), the reactions which make use of malic acid or one of its precursors ultimately predominate; the point at which inflection of the curve occurs is merely postponed.

The molar relationships between the cumulative changes in malic acid and starch at each point of observation are shown in figure 3. After 8 hours of exposure to light, 88.0 millimoles of malic acid had disappeared and 106 millimoles ($C_6H_{10}O_5$ taken as 1 mole) of starch had been formed. Accordingly, the molar ratio of the change in malic acid to that of starch at this point was 0.83; that is to say, for every mole of starch formed during the first 8 hours, 0.83 moles of malic acid disappeared. The ratios of the changes in the two subsequent intervals of 4 and 6 hours between measurements were, respectively, 0.76 and 0.95. However, the actual change in this 6-hour period was small and there was practically no change in either starch or malic acid in the final 6-hour period of exposure to light. As a result, the ratios of the cumulative changes at each point remained essentially constant.

Data from an earlier experiment on culture in continuous light (9) are plotted as a broken line in figure 3. Disregarding the high value for the ratio after the first 5 hours, during which the total change in the quantities of starch and malic acid was small and the reaction was unusually slow, there is again evidence of a considerable degree of constancy. The cumulative ratio ranges from 1.2 to 1.0 and the ratios in the two intervals between 5 and 10 hours and between 10 and 15 hours when a large amount of starch was being formed were respectively 1.04 and 0.99. It would thus appear that throughout the period beginning a few hours after these two lots of Bryophyllum leaves were first exposed to light and during which the major formation of starch and the major loss of malic acid were taking place, there was an approximately constant molar relationship between the quantities of these two substances which underwent chemical change. This relationship was such that, for every mole of starch formed, close to 1 mole of malic acid was used up.

If the transformation of malic acid into starch were quantitative and no other mechanism for the formation of starch were operative, 1.5 moles of malic acid would be needed to supply the carbon for 1 mole of starch. However, if carbon from some other source were available for the formation of starch, for example by photosynthesis, the theoretical ratio would be depressed. The observation that the ratio is not far

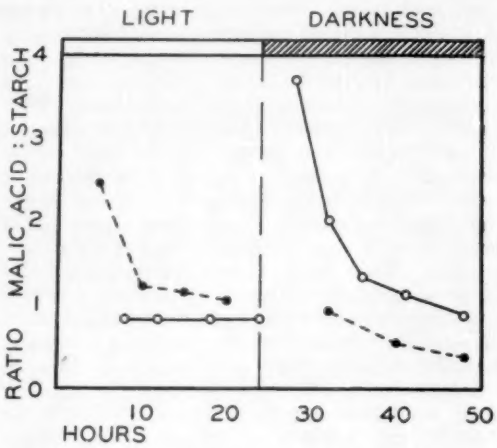

FIG. 3. Variation in the ratio at each point of observation of the cumulative change in malic acid to the cumulative change in starch in excised leaves of *Bryophyllum calycinum* cultured in water for 24 hours in light and 24 hours in darkness. Analogous data from two previously published experiments (9, 12) are plotted as broken lines for comparison.

from 1.0 during the period when starch is being produced in maximal quantity suggests that the average net effect consists in the utilization of a 4-carbon unit derived from malic acid together with a 2-carbon unit, or possibly of two 1-carbon units, for the formation of the 6-carbon unit which is finally converted into starch. The constancy of the cumulative ratio over the greater part of the period in which the leaves were exposed to light suggests that an over-all reaction of this general nature was the major one that occurred.

Nevertheless, so simple a quantitative relationship as this is not invariably observed. In an experiment carried out in 1949 (10), the increase in starch after 10 hours of illumination was 56.6 millimoles and the decrease in malic acid was 92.7 millimoles, the ratio being 1.6. It can only be assumed, in this case, that a part of the malic acid which disappeared may have been utilized in reactions other than the formation of starch. Notwithstanding this, however, the negative

correlation between the data for starch and malic acid was as highly significant as it was in the present case.

The ratios between the quantities of malic acid formed and starch used during the period of darkness are plotted at the right in figure 3. In the first 4-hour period, 28.8 millimoles of malic acid were synthesized and the loss of starch amounted to 7.8 millimoles, the molar ratio of malic acid to starch in this interval being therefore 3.7. After 8 hours of darkness, the cumulative ratio between the quantities involved had dropped to 2.0, and it subsequently fell to 0.9 at the end of 24 hours. Plotted as a broken line are data from an experiment (12) in which the behavior of the starch and malic acid was observed in leaves collected in the afternoon and cultured in darkness at 24° C. This curve is roughly parallel to that for the present experiment although placed lower on the diagram.

Obviously the situation during the early hours after Bryophyllum leaves are deprived of light is extremely complex. Carbon from some source other than starch must have been extensively drawn upon for the synthesis of malic acid in the first 4 hours and it will be recalled that this falls within the period when, according to the literature, the respiratory quotient is not far from zero and when it may even assume negative values if the air surrounding the leaves is enriched in carbon dioxide (2, 6). Under the latter circumstances, acid formation is enhanced, and Thomas and Ranson have shown that the formation of acid is distinctly greater in ordinary air containing 0.05 % of carbon dioxide than it is in air deprived of carbon dioxide. Furthermore, both Thurlow and Bonner (7) and Varner and Burrell (8) have demonstrated that malic acid quickly becomes labeled with isotope if Bryophyllum leaves are exposed in darkness to an atmosphere containing radioactive carbon dioxide. Accordingly, there is little doubt that a part of the carbon of the malic acid formed in the early phase of the exposure of the leaves to darkness was derived from externally supplied carbon dioxide. The increase in organic solids (fig 1) which occurred during the first 12 hours of darkness in the present experiment provides additional evidence in favor of this view.

In the interval between 4 and 8 hours of exposure to darkness (28th to 32nd hour), the molar ratio of the malic acid formed to the starch used dropped to 1.5 and in the period from 8 to 12 hours to 0.64. Accordingly, it would appear that the share in the synthesis of malic acid taken by substances other than starch diminished materially throughout these two intervals. After 12 hours had elapsed, malic acid reached a maximum and no significant further drain upon the supply of starch was evident. Reactions quite different in their effect upon the composition of the tissues then assumed dominance, the most easily recognized being reactions presumably involved in the general respiratory process that led to the decrease of malic acid. At this critical point, therefore, the leaves passed into a phase that became increasingly abnormal as the period of culture was prolonged.

To recapitulate the evidence in favor of such a view, the respiratory quotient rises from zero or a small positive quantity to approximately unity (1, 6, 14), utilization of starch and formation of malic acid cease, and malic acid begins to decrease. At least in the present case, glucose also began to increase. These considerations suggest that, in experiments designed for the study of the details of the reactions that occur in darkened succulent leaves, attention should be given to the preparation of the samples for the contemplated tests. The literature contains many cases in which leaves have been retained in darkness for prolonged periods in order to make sure that the starch content had been brought to its lowest possible point and that the acid content had reached a maximum. The present data suggest that such efforts may be self-defeating if the object of the tests is to study the "normal" behavior. The experience of this laboratory indicates that the adaptation of the metabolism of the Bryophyllum plant to the length of a night is so close that exposure of the leaves to darkness for much more than 12 hours at ordinary temperature brings about a situation in which the major chemical reactions that can then be demonstrated are no longer those characteristic of the behavior of leaves of plants exposed to normal conditions.

Emphasis should perhaps be placed on one further point. Although all of the evidence indicates that reactions occur in Bryophyllum leaves in which malic acid is converted into starch in light and starch is converted into malic acid in darkness, this apparently reversible interconversion is the result of a complex sequence of reactions which follow pathways that may well differ in detail in the two directions. Furthermore, there are accompanying reactions, in particular photosynthesis in light and carbon fixation in darkness, to say nothing of the correlated metabolism of citric acid, which have their effect upon the main result. The situation is thus far from simple from the chemical point of view and detailed explanation in terms of definite enzymatically controlled reactions is still a matter for future research.

Summary

Leaves of *Bryophyllum calycinum* picked at sunrise were cultured in water in light for 24 hours followed by culture in darkness for 24 hours at room temperature. The behavior of the starch, organic acids, and simple carbohydrates during the first 12 hours of each of these two periods conformed in general with that to be expected from previous studies of the changes in composition of this tissue when the leaves are exposed to the normal diurnal variation of illumination. However, in the second 12 hours of each period, the rates of the reactions which affect the starch and malic acid content became very slow and malic acid began to decrease towards the end of the period of darkness. Organic solids increased throughout the light period and for the first 12 hours of the dark period but then began to decrease. Glucose increased during the first 8 hours of the light period to a level that was maintained until the last 12 hours of the dark period when it again increased.

The conclusion is drawn that the time required at

room temperature for the major changes in starch and organic acids to occur is rather closely adapted to the period of the normal diurnal variation in illumination. The range through which the concentration of these components may move in response to the diurnal alteration of light and darkness is moderately constant for any given lot of leaves and at ordinary temperature is not increased by prolonged exposure either to light or to darkness.

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CHLORINE—A MICRONUTRIENT ELEMENT FOR HIGHER PLANTS[1]

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This article presents evidence of the essential nature of chlorine for the growth of higher plants and the proposal for its classification with the micronutrient elements. The experiments serve to support many past observations suggesting beneficial effects derived from fertilizers containing chlorine. Of particular interest are the controlled culture solution experiments of Eaton (2) and Raleigh (6) which showed highly significant increases in yields of tomatoes and cotton, and of beets, respectively, when supplied with additional chlorine. It also supports earlier work of Lipman in 1937 (4) who, after directing his attention specifically to chlorine as a growth factor for buckwheat concluded that "if chlorine is not essential, it is certainly highly beneficial."

Evidence of chlorine as a plant micronutrient offered in this paper seems conclusive beyond doubt, since inherent chlorine contaminations in culture solutions were controlled well enough to produce the nutritional disease in severe form, showing leaf symptoms of wilt, chlorosis, necrosis, and an unusual bronze discoloration which in combination are characteristic of no other known nutritional or pathological[3] disease of tomatoes. It has also been possible to maintain control at different levels of chlorine so that other significant data have been made available including yield versus chlorine supply, and the chlorine contents of roots, stems, and leaves at the various levels of chlorine deficiency. Further observations have been made

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[3] The writers wish to express their appreciation to Drs. M. W. Gardner, W. C. Snyder and A. H. Gold of the Department of Plant Pathology, who have examined these plants and their culture solutions for pathogenic organisms and have found none. Their complete description of the chloride deficiency disease will be published elsewhere.

as to rates of recovery of diseased plants after supplying chlorine, three to four days being required to arrest advance of the disease. Other points of interest are that after adding chloride ion to deficient culture solutions, new growth appears healthy and remains so and moderately injured leaf areas progressively recover from chlorosis and resume growth; but leaf areas where chlorosis has progressed into bronzing, although they regain some of their original color, remain static and do not expand.

The present data on the chlorine nutrition of plants were an outgrowth of intensive work on cobalt, which was of interest because of its recognized need in the life of animals, but whose essentiality has not been critically demonstrated for plant life. In beginning this work, it appeared that cobalt offered a special challenge for investigation, particularly because of the very small amounts known to be involved. Consequently, nutrient salts of analytical reagent grade were treated chemically to remove cobalt as the sulfide along with copper or iron sulfides. As an improvement in the degree of sensitivity of detection and estimation of cobalt, a known amount of high specific activity radio-cobalt was added to the salt solutions undergoing purification, and its residual activity was used as an indicator of the degree of removal of this element. The most satisfactory removal of cobalt from culture solutions was attained through alkaline sulfide co-precipitation with either copper or iron salts as carriers. Measurements of the residual radioactivity of Co showed that the purified nutrient salts contributed less than 2×10^{-4} micromoles (0.01 μg) cobalt per liter of culture solution. Presumably the same process which allowed this degree of removal of cobalt would have similar merit for other sulfide precipitable metals. It is expected that details of these procedures will be reported elsewhere.

Using the salts thus purified, experiments were designed to test plant growth responses to cobalt at a level of 0.085 micromoles (5 μg) per liter of culture solution. One of the striking observations of the first experiment was that provision of cobalt at 5 μg per liter (also 5 μg per plant) resulted in a 50 % increase in shoot yield—statistically significant at the 5 % level, strongly suggesting cobalt as a growth factor. However, in following experiments it became apparent that culture solutions made from these salts, even with additions of cobalt up to 1.7 micromoles (100 μg) per liter, would not allow plants to grow satisfactorily as compared to growth made on similar culture solutions of technical grade nutrient salts. Because of this, the question was raised as to whether purification procedures had resulted in the addition of a toxic material or whether significant amounts of another unknown element necessary for growth of the tomato plant had been eliminated. There also remained the question as to why the 5 ppb cobalt addition of the previous experiment showed an increased yield and it was speculated that the cobalt salt itself might have contained the unknown growth factor. Whatever the case, it was obvious that further progress could not be made until it was found how to grow plants on these purified culture solutions. The idea of toxicity seemed unlikely. The only toxic materials added to the salts during purification were copper salts and H_2S, and auxiliary experiments with added H_2S or copper did not produce these symptoms. Also, similar separations had been used many times in our laboratories for producing plants deficient in Mo, Cu, Zn, Mn, or Fe without previous evidence of toxicity. Therefore, it was assumed that the stock greenhouse salts contained a growth factor which was not present in the purified salts in sufficient quantity to meet the needs for growth of the tomato plant and work was directed toward its identification. Pending identification, it became known as "element X."

As a first step in localizing "element X," greenhouse stock salts (KNO_3, KH_2PO_4, $Ca(NO_3)_2$, and $MgSO_4$) were examined separately for presence of the growth factor by adding the single salts to complementary groups of culture solutions otherwise compounded from the purified salts known to give rise to the disease. For comparison, tomato plants were grown on both the purified cultures and stock greenhouse cultures. As before, diseased plants developed on cultures made entirely from the purified salts and healthy plants grew on the technical grade salts. However, healthy plants were also obtained when either KNO_3 or KH_2PO_4 from the technical stock was added to the purified cultures. Addition of technical grade $Ca(NO_3)_2$ or $MgSO_4$ did not correct the disease. From these experiments it was concluded that the growth factor was contained in each of the technical grade potassium salts. The question was then raised as to the possibility of a significant association of the unidentified growth factor with the potassium salts. Potassium chloride is the parent raw material of most other potassium salts of commerce and in the processing of crude KCl a great many important contaminants are recognized. However, for our purposes, the task of analyzing for all the many possibilities seemed too great, particularly after unfruitful exploratory analyses of the potassium salts with the hydrogen flame spectrophotometer failed to reveal any prominent differences between those having and those not having the growth factor.

Consequently, a supply of the U.S.P. KNO_3, now known to contain the growth factor, was taken from the technical stock and subjected to fractionation by a variety of chemical processes, including recrystallization. The latter method was the only one which showed promise of concentrating the growth factor. For comparison with the U.S.P. KNO_3 which contained the growth factor, a sample of commercial fertilizer grade KCl [4] was similarly processed. Tests of the mother liquors and the recrystallized salts for presence of the growth factor were then made by growing tomato plants in culture solutions wherein the potassium component was provided from the above fractions of potassium salts; otherwise, the culture solutions were prepared from the purified salts.

[4] Supplied by the Pacific Guano Company as representative of commercial KCl produced at Searles Lake, California.

Thus, healthy plants would show the presence of "element X" in the substituted potassium salt.

For these tests, a new lot of reagent grade salts was purified as before, since the original stock had become depleted. For five weeks of the growth period it was a matter of concern that no evidence of the disease assigned to "element X" deficiency appeared, and it was concluded that the elaborate purification procedure for metal separation was not responsible for the purity of the earlier batches of salts. The plants were continued for another week and having learned from past experience how to detect early stages of the disease, we were rewarded by recognition of its appearance in mild form in some of the cultures. From the cultures showing the disease we concluded—though not very firmly—that recrystallized KNO_3 had lost "element X" while its mother liquor had not. It also seemed reasonable to conclude tentatively that the growth factor was supplied by a) the fertilizer grade KCl; b) the recrystallized KCl; and also c) the mother liquor from the recrystallized KCl. Thus, it appeared that chloride ion might be the missing growth factor. Further evidence from the experiments to be discussed proved that it was.

Meanwhile, ten single tomato plants, each on a separate culture solution composed of our earlier set of salts of low "X" content were grown with the hope of procuring seed more dilute than normal with respect to "element X." However, blossoms would not set, except on one plant which produced two single fruits. By comparison, another set of tomato plants grown at the same time upon the technical grade nutrient solutions blossomed profusely and produced a heavy set of fruit. The latter plants were judged to be high yielding as compared with commercial standards.

Thus, it became known that the first lot of purified salts was too low in "element X" to meet the nutritional requirement demanded of it for the continuation of the life cycle of the tomato plant, simply because blossoms would not mature or set fruit. To test the now very strong suspicion with respect to chlorine as "element X," a large scale experiment was designed, but in this instance salts were pre-treated with silver ion to remove the silver precipitable halides, after which the second purification for heavy metals was undertaken.

Materials and Methods

Experimental Procedure Testing the Requirement for Chlorine in the Growth and Development of the Tomato Plant: On the assumption that chlorine was the growth factor, an experiment of 64 cultures was designed to find the levels required for the adequate chlorine nutrition of the tomato plant. With the exception of 4 cultures using technical grade, all salts used for making the cultures were subjected either to recrystallization (especially $(NH_4)_2HPO_4$) or to halide precipitation with $AgNO_3$, the excess silver being removed subsequently by coprecipitation with copper as the sulfide.

The culture solutions contained the following nutrient ions in millimoles per liter: Ca, 4; Mg, 1; K, 6; NH_4, 2; H_2PO_4, 2; SO_4, 1; and NO_3, 14. They were purified as salt solutions of $Ca(NO_3)_2$, KNO_3, $Mg(NO_3)_2$, $(NH_4)_2HPO_4$, and K_2HPO_4. H_2SO_4 was then added to the phosphate salts to bring them to the dihydrogen equivalent. The relative proportion of the ammonium to nitrate ion as nitrogen sources was chosen because with this proportion the hydrogen ion concentration of the solutions remained reasonably near a pH value of 6 throughout the growth period.

The amounts of micronutrients used are generally expressed in micromoles per liter or per culture. The odd amounts reflect the customary round number concentrations traditionally given in parts per million or parts per billion. Six micronutrient elements were added in micromoles per liter as follows: Fe, 86; B, 46; Mn, 9.1; Zn, 0.76; Cu, 0.31; Mo, 0.10. The chlorine treatments were made in series, with the lowest concentration being the chlorine remaining in the salts after the purification process. The residual chlorine was determined by analysis to be 3.0 micromoles per liter. Considering the residual chlorine found by analysis, the total chlorine in the other members of the series was respectively 3.7, 6.5, 20.6, and 105 micromoles per liter. Cultures made from the technical grade salts contained 11.7 micromoles per liter.

The added chlorine supplements were taken from 6 different sources, namely: 1) reagent grade KCl; 2) technical grade KCl; 3) recrystallized technical grade KCl; 4) mother liquor from the recrystallized KCl; 5) reagent grade $CaCl_2$; and 6) reagent grade $MgCl_2$. Each supplement was duplicated, making a total of 12 cultures having received the same amount of chlorine. Later on, it became clear that the symptoms were related specifically to the amount of chlorine supplied to the cultures irrespective of source.

Iodine and bromine were also tested for possible interrelations with chlorine, but the experiments were less elaborate, having but two replications per treatment. To the 3 micromoles of residual chlorine per liter, bromine was added as KBr at levels of 0.31 and 7.8 micromoles per liter, and iodine as KI at levels of 0.20 and 4.9 micromoles per liter. Although there were but two replications for each treatment, the duplicate cultures responded uniformly as judged by visual observation. The plant response to iodine or bromine was unquestionably different from that obtained with chlorine at equivalent levels. Both iodine treatments appeared to be toxic, and the eventual yields were lower than for any other plants. Also, yields of plants treated with 4.9 micromoles of iodine were slightly lower than those receiving 0.20 micromoles per liter. At early growth stages the higher iodine treatment showed the more severe effect.

Special consideration was taken to avoid possible sources of chlorine contamination while setting up the experiment. For example, our laboratory custom of washing all beakers with 10% HCl as part of the cleaning procedure for micronutrient element studies received special attention. As a part of the regular rinsing procedure a jet of redistilled water was sprayed around the walls of the beakers. When about

50 ml of water had accumulated, it was poured off and and its pH was tested with the glass electrode. This rinsing process was repeated until the pH of the discarded rinse water was the same as the original. In all subsequent experimentation 1 % HNO_3 was substituted for HCl.

Plant Culture Method: Marglobe tomatoes were germinated over Pyrex glass trays on cheesecloth. They were watered with a one tenth strength culture solution without added micronutrients. They were transplanted in the cotyledon stage to Pyrex glass beakers of 4 liters capacity having paraffined plaster of Paris covers. The roots were shielded from light by double lined cloth skirts, white outside and blue denim inside. Solutions were aerated continuously. Five plants were set out on each culture and these were thinned to the four most uniform, after one week.

Molar stock solutions of the macronutrient salts were purified by the precipitation of halides with silver nitrate and subsequent removal of the excess silver by an alkaline copper sulfide co-precipitation. Heated stock solutions, to which a small excess of silver nitrate was added, were allowed to cool overnight and then filtered. The filtrate was heated to boiling and treated with 10 mg copper (as $CuSO_4$) and 1 gm of low-halide $CaCO_3$, per liter of solution. Hydrogen sulfide was then passed into the solution and continued for half an hour while the solution slowly cooled. After cooling overnight, the solution was again filtered, brought to a boil, and water pumped nitrogen bubbled through the boiling solution to remove excess hydrogen sulfide. Solutions prepared in this manner are sufficiently low in halides and heavy metals including iron, manganese, zinc, copper, and molybdenum to be used in solution culture studies involving these elements.

Recrystallization of reagent grade salts is also effective in removing halides and has been used in some of these studies. The technique involves the use of the first fraction of crystals as the halides appear to concentrate in the supernatant.

Halide determinations on solutions were done by one of two methods depending on the amounts present. For very dilute concentrations or where size of samples was limited the microdiffusion method of Conway (1) was used. For larger amounts, in excess of 10 micromoles halide per sample, a modification of the potentiometric method of Kolthoff and Kuroda (3) was used. Plant samples were ashed at 500° C, after treatment with freshly prepared low-halide calcium oxide as recommended by Piper (5). Complete details of analytical methods and results will be reported in another paper.

Growth and Development of the Tomato Plant at Different Levels of Chlorine Supply: The reward for the above precautions was an exceedingly uniform set of plants in all 64 beakers of the series. No differences in health or growth, as judged by visual observation, could be detected in any of the cultures until 24 days after transplanting. On that day the first signs of abnormality appeared as the characteristic "low chlorine" wilt of leaf tips, as manifested by the tomato plant. The treatments affected were a) each of the four pots having received iodine supplements at lower and higher levels; b) each of the two pots receiving the lower bromine supplement (1.25 micromoles Br per culture) and c) two of the four replicates to which no halide was added but which contained as an unremoved impurity, 12.0 micromoles chlorine per 4 liters of culture solution. Two days following the first sign of the deficiency disease all four of the lowest chlorine cultures showed similar symptoms. Two days later the two higher level bromine treatments (31.2 micromoles Br per culture) became affected. Also on this last date all of the 12 treatments having received the lowest added supplement of chlorine (2.8 micromoles per culture) acquired the wilt symptom. None of the remaining cultures at higher chlorine levels was affected. It appeared at that time that 31.2 micromoles of bromine was as effective as 2.8 micromoles of chlorine in delaying onset of the disease.

The next set of plants to show the disease was the 12 cultures at the next higher chlorine level (14.1 micromoles added chlorine per culture). Upon the 10th day following the first indication of disease on the lowest chlorine level, each and every one of the cultures which received 14.1 micromoles chlorine became affected, but at the same time none of the cultures having chlorine levels above this showed the disease, nor did they show it during the remaining ten days of the experiment.

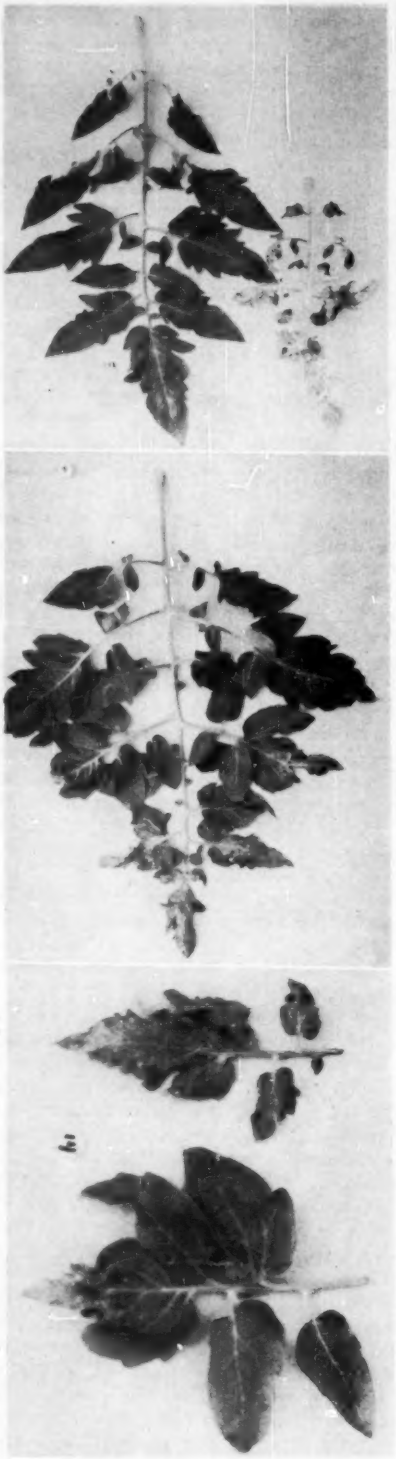
Recovery of Chlorine Deficient Plants Following Addition of Reagent Grade KCl: It appeared that the delaying of symptoms was related to addition of chlorine and not to the kind of chloride salt containing it, that is reagent grades $CaCl_2$, $MgCl_2$, and KCl, technical grade KCl, mother liquor KCl, and recrystallized KCl. Thus it was considered worth while to divide the 12 diseased replicates which had been supplemented with 2.8 micromoles chlorine per culture, and from 6 sources of chloride salts, into two lots by selecting one culture from each of the salt types. One lot was given an additional supplement of 352 micromoles per culture as reagent grade KCl, i.e., approximately 3 parts per million in the culture solution. All six of the treated cultures recovered completely, the last symptom of wilt disappearing on the sixth day after treatment. By contrast, the six cultures which did not receive the extra chlorine supplement progressed in their symptomatology into chlorosis, bronzing, and necrosis of leaves. They also became most obviously restricted in growth. At harvest time, 13 days after treatment with KCl, the treated plants showed a large difference in yield which was statistically significant at better than 1 % with respect to leaves, stems, roots, or total yield. The weights of the recovered vs. diseased plants were: 65.3 vs. 42.7 for leaves, 58.6 vs. 41.4 for stems, and 23.4 vs. 15.0 grams dry weight for roots.

Recovery of Plants Following Addition of NH_4Cl Prepared by Cold Gas Purification: Although the above experiments seemed thoroughly con-

clusive that the element responsible for relieving symptoms of injury was chlorine, there was a question brought forth that some other element might have fortuitously followed chlorine throughout the chemistry of the manufacturing processes for each of the salts used as a source of chlorine and that this postulated contaminating element might be the effective one rather than chlorine. Therefore, a new salt of ammonium chloride was prepared from ammonium hydroxide and hydrochloric acid, by a cold distillation process. Nitrogen was passed through concentrated reagent grade HCl and bubbled into glass redistilled water until the resultant solution was approximately 0.1 *N* with respect to HCl. Similarly, nitrogen was passed through reagent grade NH_4OH, and discharged into the 0.1 *N* HCl solution to neutralize the latter. Aliquots of the NH_4Cl so prepared were analyzed for chlorine content. This method of preparation was designed to obviate inclusion of heavy metal and other nongaseous contaminants which might have been present in the aqueous stocks of reagent grade NH_4OH or HCl.

To test the effectiveness of the NH_4Cl in removing the symptoms under observation, the 12 cultures of injured plants having received an added 14.1 micromoles chlorine per culture were divided into two sets in the same way as described for the recovery set just discussed. As with the earlier recovery experiment in which reagent grade KCl was used, the added amount of chlorine was 352 micromoles per culture (approximately 3 ppm Cl in the culture solution), but derived from the cold gas-purified NH_4Cl. This recovery experiment was started 10 days before harvest. Six days later 23 of the 24 affected plants had recovered from symptoms. Nine days later all 24 had recovered completely. By contrast, all 24 plants on the 6 cultures which did not have the NH_4Cl supplement, progressed into symptomatology of wilt in severe form, and 23 of them acquired the advanced symptoms of bronzing. Effectiveness of this chloride treatment was also shown in an increased weight of the chloride treated plants. The dry weights of the recovered versus the non-recovered plants were: leaves 71.4 vs. 58.8, stems 63.2 vs. 54.6, roots 27.4 vs. 22.1 grams dry weight. The increased yield was significant in each instance at the 1 % level.

Recovery of Chlorine Deficient Tomato Plants through Injection: From the four replicates of lowest halide plants, one culture was selected for recovery experiments through injection. There were four plants growing on the single culture. All plants were severely injured and in the stage of advanced leaf necrosis. Three of the four plants were injected respectively with a) redistilled H_2O, b) 0.003 *N* KNO_3, and c) 0.003 *N* KCl. A hypodermic needle was inserted into leaf-stem junctions directed steeply downward to bring its point within the pithy region of the stem. Five junctions were punctured, and a total of 0.056 micromoles (2 μg) of chlorine were thus introduced within the stem tissue of one plant. As controls, similar volumes of solution were injected into two of the other plants, one being KNO_3



and the other H_2O. The fourth plant was not injected.

Three days after injection, growing tips of the chloride injected plant appeared to grow without injury, whereas all other plants showed necrotic spots. This early pattern continued. However, further injections of equal volume were made 2 weeks later using H_2O, 0.1 *N* KNO_3 and 0.1 *N* KCl. The chlorine administered at this time was 0.56 micromoles (20 μg). New growth developed rapidly and in a seemingly normal fashion only with the plant injected with KCl. Since that time, many other recovery experiments have been run, and for this reason, we are assured that the reactions shown by the KCl injected plant are typical, and the result of chlorine being added. Older leaves badly affected did not recover, but mildly affected ones did. The pattern of the general recovery is of sufficient interest, that some of its features are included in figure 1.

YIELD OF TOMATO PLANT AS A FUNCTION OF CHLORINE SUPPLY: The tomatoes were grown for 45 days after transplanting, then harvested. The plants were separated into three fractions—leaf blades, stems including petioles, and roots, and were weighed after oven drying at 70° C. In figure 2 the average dry weight of each plant part per culture is plotted against the known total amount of chlorine supplied per culture in micromoles. The total chlorine supplied includes 3.0 micromoles per culture as residual salt contamination which could not be removed in the purification process, but which was shown upon analysis for chlorine. In figure 2 the points shown by open circles are the ones from cultures having had halogens removed either by salt recrystallization or by precipitation with silver ion and the excess silver subsequently removed as Ag_2S coprecipitated with CuS. The black points are the yields of leaves, stems and roots obtained from plants grown on the technical grade salts regularly used for making culture solutions in our greenhouses. These latter salts were analyzed for chlorine and were shown to contain enough impurity to provide 47 micromoles of chlorine for the 4 liters of the culture solution. As earlier suggested, the content of "element X" in the greenhouse salts was sufficient to give good growth by customary standards. From these data (fig 2) it appears that chlorine supplements even to these greenhouse salts might have resulted in significantly greater yields, depending upon care taken to exclude chlorine contaminations from other sources.

FIG. 1. Leaf development under adequate and limiting chlorine supply and recovery following subsequent application.

A. Normal leaf (left) produced after injecting plant with 22 μg Cl. This photograph was taken 22 days following the first injection of 2 μg and 8 days after the second injection of 20 μg Cl. This leaf was the 14th, counting from the base. Cl-deficient leaf (right), 8th from the base of the plant injected with KNO_3. This leaf is very similar to the 8th leaves on the water injected plant and on the uninjected control. For the 8th leaf of the KCl injected plant compare figure 1-B.

B. Recovered leaf, 8th from the base of the KCl injected plant. The terminal leaflets were moderately bronzed at the time of injection and never recovered. Some leaflets only mildly bronzed, grew basipetally, but not acropetally beyond the bronzed area. The tips of the leaflets which were wilted but unbronzed, recovered turgor, but failed to expand. Compare with 8th leaf, figure 1-A, right.

C. Leaflets from water (upper) and KCl (lower) injected plants. These are corresponding leaflets taken from the 8th leaf. The lower leaflet of this figure is the one shown in figure 1-B, the 4th down on the left. The basipetal expansion as contrasted with restricted growth of the tip is very characteristic of the recovery pattern of mildly bronzed leaves of Cl-deficient plants after recovery.

DISCUSSION

The chlorine requirement is not small as compared to other micronutrients, for in the leaves of tomato plants suffering from chlorine deficiency disease, the chlorine concentration is in the order of 7 micromoles per gm dry weight (250 ppm). Data bearing on this point will be presented in another paper. By comparison, the presently recognized micronutrient which is required in the least amount is molybdenum and plants showing an equivalent degree of visual stress by reason of molybdenum deficiency would have in the order of 1.4×10^{-3} micromoles per gram (0.1 ppm) or less of molybdenum. Thus, on a mole basis, the minimal quantity of chlorine required in plant tissue is several thousand times greater than for molybdenum. Plant requirements for Fe, B, Mn, Zn, and Cu are intermediate, and reasonably within the order given.

In recognizing chlorine as a plant nutrient, it is with the realization from this experiment that Br can substitute at least partially in plant functions normally dependent on Cl, in a way reminiscent of the sparing effect of Na for K or Sr for Ca. Whether or not Br can substitute completely for Cl must remain a subject for future investigation.

SUMMARY

1. A severe nutritional deficiency disease was observed in tomato plants when the known halide supply was limited.

2. The nutritional deficiency is visibly characterized in its early stages, by a wilting of leaflet blade tips; progressively, by chlorosis, bronzing and necrosis basipetally in areas proximal to the wilting.

3. Severely diseased plants failed to produce fruit.

4. Growth was correlated with chlorine supply in the culture solution.

5. Adequate additions of chlorine as chloride to culture media entirely prevented the disease; severely deficient plants resumed satisfactory growth after chlorine was applied to the culture solution or through injection into stems.

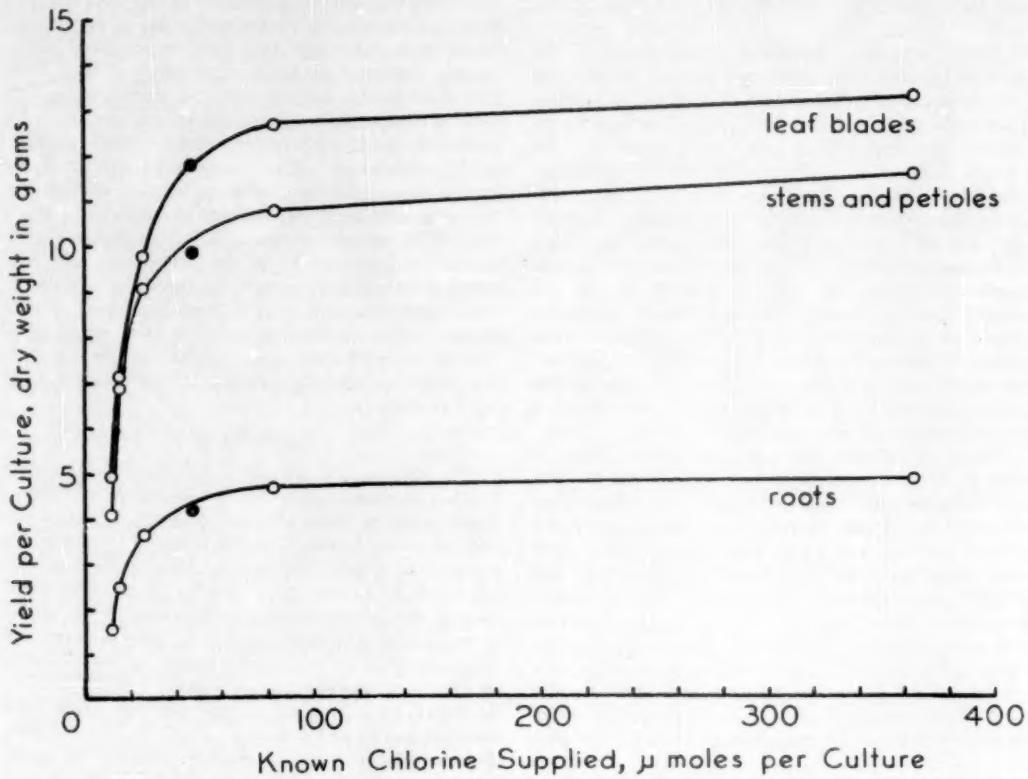


FIG. 2. Relation between yield of plant material and total known chlorine supply. Open circles indicate values from repurified salt solutions; closed circles, from technical grade salts.

6. Bromine appears to complement chlorine when supplied at about ten times the required chlorine levels. Possible effects of iodine are difficult to assess because of its toxicity.

It is concluded that chlorine is a nutrient element, certainly the naturally occurring essential halide.

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GROWTH INHIBITOR ACTIVITY IN XANTHIUM IN RELATION TO PHOTOPERIODISM[1]

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Indirect evidence has indicated that a relationship exists between auxin level and the flowering phenomenon in various plants. Hamner and Bonner (8) found that the application of indoleacetic acid in lanolin decreased the amount of flowering response in the short-day plant Xanthium grown under conditions of short photoperiod. Bonner and Thurlow (4) and van Senden (18) showed that auxin applied to the leaves of short-day plants inhibited flowering. Furthermore, when auxin antagonists were applied by Bonner (3), these substances were found to promote flower initiation in short-day plants under daylengths unfavorable for flowering. Khudairi (10) found that ethylene chlorohydrin, which is thought to lower the auxin content of plant tissue (13, 9, 15), promoted flowering in Xanthium under conditions unfavorable for flowering. Galston (7) showed that triiodobenzoic acid apparently acts in opposition to auxin and when applied to photoinduced short-day soybean plants considerably increases the number of flowers.

Long-day plants have been shown to have a response contrary to that of short-day plants, i.e., applied auxins favor flowering. Leopold and Thimann (12) found that low concentrations of naphthaleneacetic acid and indoleacetic acid promoted flowering in long-day barley, while short-day teosinte showed no stimulation. Both plants showed inhibition of flowering at high concentrations. Clark and Kerns (5) induced flowering in the pineapple by application of substances active as auxins, although high concentrations prevented flowering. Van Overbeek et al (17) and van Overbeek and Cruzado (16) suggest that in the pineapple flowering under natural conditions takes place as a result of an increased level of free auxin. Roberts (14) reported that a crystalline substance isolated from various flowering plants had antiauxin properties.

Attempts have been made to relate auxin analyses of Xanthium leaves to photoperiodic responses, but these have been unsuccessful. In new experiments by the authors it was found that a growth inhibitor which is present in large amounts interferes with auxin analysis. The first observations on the inhibitor were reported by Bonde (1), and effects of the inhibitor on growth and other physiological relations were reported by Bonde and Khudairi (2). Some relations of the inhibitory substance to photoperiodic responses are to be presented in this paper.

[1] Received August 18, 1954.

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MATERIALS AND METHODS

Xanthium pensylvanicum Wallr. (*X. saccharatum* Wallr.) (6) seeds[4] collected in the vicinity of Chicago, Illinois, were used in these investigations. The plants were grown in 4-inch pots under greenhouse conditions and were kept vegetative by being given supplementary light of about 50 fc from sunset until 2:00 A.M. The method used for the extraction of the inhibitor were described by Bonde (1). Usually 5 gm of leaf blades was extracted as a sample, and 0.5-ml aliquots of the total 25 ml of extract were tested for inhibitor activity, using the Avena coleoptile curvature test (19). The aliquot to be tested was mixed with a known amount of indoleacetic acid (50 μg IAA/l agar) and evaporated to dryness, and the residue was mixed with 2 ml of 1.5 % agar. The agar blocks were left on the Avena plants for 2.5 hours, this time interval giving proportional results, and the curvature in degrees was determined from shadowgraphs. The percentage of inhibition produced by a certain aliquot of inhibitor solution when added to the IAA was determined by using the difference in degrees between the curvature of the Avena coleoptiles with IAA alone and with IAA mixed with inhibitor. The results are reported as percentage of inhibition.

Different parts of vegetative plants were tested for comparative amounts of inhibitor activity. Five gm each of young expanded blades, older expanded blades, petioles, stems, and green burs were extracted with ether for 2 hours at 0° C, and the extracts were tested as previously described. The percentage of inhibition of 50 μg/l IAA by the same amount of extract from each of the various tissues is shown in figure 1. There was very little inhibitor activity in the petioles, stems, and burs as compared to the leaf blades. The highest amount was present in the unexpanded blades (95 % inhibition), while the first three expanded blades had less (60 % inhibition), and the fourth expanded blades still less (30 % inhibition). The petioles, burs, and stems gave less than 20 % inhibition.

In order to compare the inhibitor activity of leaves of different ages, three leaf blades of each of the following sizes were extracted with ether for 2 hours at 0° C: unexpanded leaf blades 1 to 2 cm^2 in area, 5 to 10 cm^2, and 20 to 30 cm^2; and the first, second, third, fourth, and fifth fully expanded blades (from the top to the bottom of the plant), the average area of each being about 45 cm^2. In figure 2 it may be seen that the maximum inhibition on a whole leaf blade basis

[4] Specimens of the plant used in these studies have been filed at the herbarium of the University of California, Los Angeles.

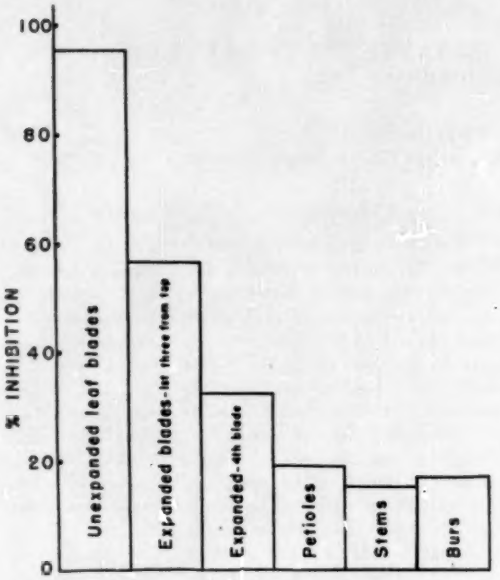


FIG. 1. Relative inhibitor activity of extracts of equal fresh weights of different parts of Xanthium plants. Aliquots corresponding to extract of 0.1 gm tissue were used with 0.1 μg IAA in the Avena test. The ordinate indicates % inhibition of curvature in the test.

was produced by extract from the unexpanded leaves 20 to 30 cm² in area and that there was a continuous decrease in inhibition with increase in age of the expanded leaves, the fifth expanded leaf showing no inhibition with the amount of extract used.

A similar study of inhibition on a fresh weight basis was carried out, and the data are presented in figure 3. In each case extract of 0.12 gm fresh weight of tissue was combined with 0.1 μg IAA in 2 ml of 1.5 % agar. A similar amount of an extract of terminal buds was also tested. Figure 3 shows that except for the buds the youngest leaf blades show the most inhibitor activity per gram fresh weight and that with increasing age there is a steady decline in the inhibitor activity of the extracts of the leaves. The buds have less activity than the expanding leaves and more than the expanded ones.

Figure 4 illustrates the inhibitor activity of extracts brought out by figure 2 in another manner. Seven lots of two Xanthium plants each were defoliated at the beginning of the experiment except for a single leaf on each plant about 15 cm² in area. The leaves of successive lots were removed and extracted at 0, 0.5, 1, 2, 4, 6, and 10 days after the beginning of the experiment. As may be seen in the figure, the inhibitor activity rises to a maximum on a leaf basis between the fourth and sixth days after attaining the 15 cm² size. Between the fourth and sixth days the leaves had reached an area of about 30 cm², at which size a maximum is also shown in figure 2. After that time a decrease in the inhibitor activity becomes evident.

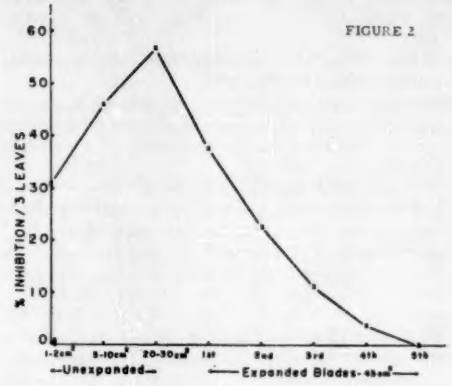
Further experiments were performed to determine whether changes in the inhibitor activity of extracts took place when leaves were exposed to increasing numbers of short days, whereby the plants were induced to flower. All the expanded leaves were cut from vegetative plants, leaving only one unexpanded leaf of the same size on each plant. Lots of six plants each were given 0, 1, 2, 4, 6, and 8 short days (dark period 16 hours long) respectively, the first group being the control maintained under long days. In each case the final short-day treatment was given on the day preceding the extraction of the leaves. Extract of 0.08 gm of fresh leaves from each lot was combined with 0.1 μg of IAA in 2 ml agar in the Avena test. Figure 5 shows a decrease in inhibitor activity with increase in the number of short days up to the second day. From the second to the fourth day there is a slight rise in activity, after which it decreases to the eighth day. The differences are not great, but a downward trend in inhibitor activity of extracts with increasing number of short days seems apparent.

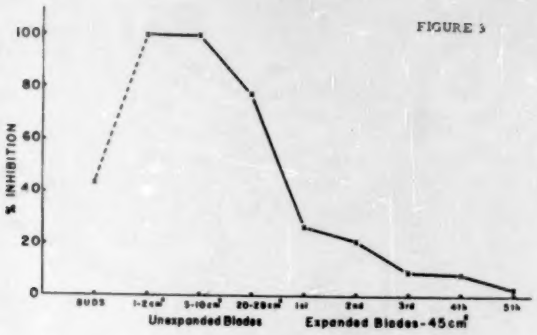
DISCUSSION

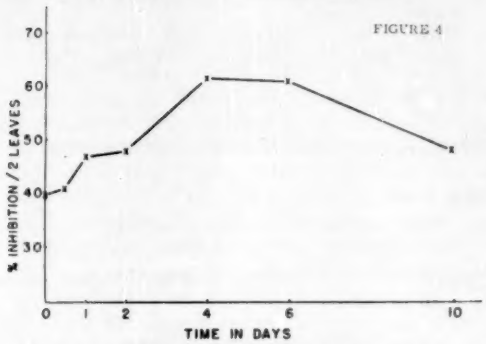
It was of interest to study the inhibitor activity of extracts of various plant parts, of leaves of different ages, and of leaves which had been subjected to photoperiodic induction treatment in an effort to determine the relation of the inhibitory substance to the photoperiodic reaction in Xanthium. It is known that reception of the photoperiodic stimulus in Xanthium is primarily limited to the leaf blades (8), and it was found in this study that the leaf blade was the plant part with the highest inhibitor activity. Furthermore, the leaves which were found to have the highest activity were the same as the ones found by Khudairi and Hamner (11) to be most sensitive to the photoperiodic stimulus, the sensitivity curve being almost parallel to that of the inhibitor activity for the leaves of various ages. The inhibitor activity was found to have an apparent relation to photoperiodic induction in that it decreased in the leaf blades when the plant was induced to flower, and activity decreased with increasing number of short days. This relationship, however, may only be the result of the complicated physiological changes in the plant associated with the change from the vegetative to the flowering state and thus may bear no causal relationship to the initiation of flowering.

The fact that the inhibitor activity is highest in the leaves most receptive to the photoperiodic stimulus is in accord with the observation that auxin inhibits flowering in Xanthium, inasmuch as the effective auxin concentration is presumably low in such leaves. However, the apparent decrease in inhibitor activity associated with the induction of flowering does not fit the concept that flowering results when a high inhibitor concentration lowers the auxin level sufficiently.

It should be pointed out that with the use of the Avena coleoptile curvature test differences in inhibitor activity could mean differences in amount of







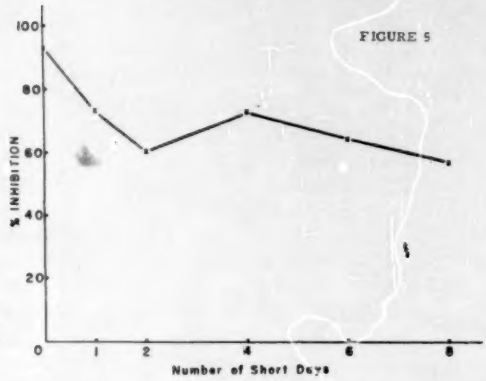


FIG. 2. Relative inhibitor activity of extracts of whole leaf blades of different ages. Three blades of each age were extracted with ether, and extract was made up to 25 ml. Aliquots of 0.4 ml were used.

FIG. 3. Relative inhibitor activity of extracts of equal fresh weights of leaf blades of different ages. Aliquots corresponding to extract of 0.12 gm tissue were used.

FIG. 4. Relative inhibitor activity of extracts of series of whole leaf blades extracted at different times after removal of all but single leaf from plants. Two leaf blades were extracted at end of each interval, and extract was made up to 25 ml. Aliquots of 0.6 ml were used.

FIG. 5. Relationship between inhibitor activity of extracts of leaves of the same size and the number of short days (16-hr dark periods) given to the plants previous to extraction. Aliquots corresponding to extract of 0.08 gm fresh tissue were used.

auxin extracted from the tissue at the same time. The high inhibitor concentration in the leaves, however, more likely obscures variations in measurable auxin levels.

SUMMARY

1. Growth inhibitor activity of extracts of various fresh Xanthium plant parts was greatest in extracts of leaf blades, unexpanded blades showing more activity than fully expanded ones. Extracts of petioles, stems, and burs showed relatively little activity.

2. When equal fresh weights of tissue were compared, extracts of the youngest leaf blades had the highest inhibitor activity, with the activity decreasing markedly with increase in age of the leaves. When whole leaf blades were compared, extracts of leaf blades 20 to 30 cm² in area showed the highest activity.

3. When inhibitor activities of extracts of a series of leaf blades which had been subjected to different numbers of short days were compared, the activity was found to decrease gradually with increase in the number of short days to which the leaves had been exposed.

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THE FLOWERING OF SUNFLOWER EXPLANTS IN ASEPTIC CULTURE[1, 2]

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The aseptic culture of entire flowers, floral parts and flower initiating explants has been accomplished by several investigators. La Rue (1, 2) maintained whole flowers and floral parts in a healthy condition for long periods of time on a synthetic medium. Loo (4) succeeded in culturing excised stem tips of *Cuscuta campestris* upon which flowers developed despite the absence of leaves and roots. In the same year Loo (5) reported the culturing of *Baeria chrysostoma* through its entire life cycle. This plant is a small desert annual which can flower as soon as 19 days after germination.

Since it has been shown that isolated plant parts as well as entire plants can initiate flowers in sterile culture, a means is available to study the influence of the different organs of the embryo on reproductive growth. Using the sterile technique it is possible to culture the isolated plumule or the plumule in combination with one or all of the other organs of the embryo. As a starting point, the study of the effect of cotyledonous tissue on the development of the plumule was selected.

Since light intensity exerts such great influence in plant growth, it was felt that culturing the explants under different light intensities might yield further information on the cotyledon to plumule relationship. Therefore, explants of sunflower plumules alone or plumules with different quantities of attached cotyledonary tissue were cultured aseptically under various light intensities.

Materials and Methods

The explants used were excised from 5-day-old seedlings of *Helianthus annuus* L. var. Mammoth Russian. The seeds were soaked 4 hours in tap water, sterilized with $HgCl_2$ (1:1000) for 10 minutes and rinsed three times with sterile distilled water. They were then transferred into sterilized Petri dishes containing several layers of moistened filter paper where they germinated in the dark.

The explants grew in cotton-stoppered 30 × 200 mm Pyrex test tubes containing 20 ml of substrate. The basic medium employed was a slight modification of that described by White (7). The medium consisted of 200 mg $Ca(NO_3)_2$, 200 mg Na_2SO_4, 80 mg KNO_3, 65 mg KCl, 16.5 mg $NaH_2PO_4 \cdot H_2O$, 4.5 mg $MnSO_4$, 1.5 mg $ZnSO_4$, 1.5 mg H_3BO_3, 0.75 mg KI, 360 mg $MgSO_4$, 2.5 mg $FeC_4H_4O_6$, 3.0 mg glycine, 0.5 mg nicotinic acid, 0.1 mg pyridoxine, 0.1 mg thiamine, 20 gm sucrose, and 4.5 gm of Difco Bacto-Agar dissolved in enough double distilled water to make a total volume of one liter. Duplicates of all experiments were also run in which sucrose was omitted from the medium.

The preparation of the explants and their culture were carried out aseptically. Flamed implements were used and all excising was done on a flamed microscope slide under a dissecting microscope. The microscope was fitted with an ocular micrometer and had a magnification of 10 times. One cotyledon and all tissues below the cotyledonary node were removed from all

[1] Received August 23, 1954.

[2] Papers from the Department of Botany and Plant Pathology, Ohio State University, No. 546.

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explants. In addition, in some explants all or part of the remaining cotyledon was cut away. Thus the explants consisted of plumules alone or plumules with varying amounts of attached cotyledonary tissue. Series consisting of plumules alone and plumules bearing $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{3}$, $\frac{1}{2}$, and 1 entire attached cotyledon were prepared.

Each series was divided into four sub-series and each of the sub-series was subjected to a different light treatment. These treatments consisted of dark, or approximately 10, 400, and 800 fc of Daylight fluorescent light. Light intensities were measured with a Weston Illumination Meter, Model 603. The data obtained were based upon 15 to 40 replications within each sub-series. The temperature was maintained at 26° C and the photoperiod was 16 hours.

Results

Flowering was chiefly confined to explants with small pieces of attached cotyledonary tissue growing under 400 or 800 fc intensity. Explants with larger pieces of attached cotyledons flowered infrequently or not at all under these intensities. Flowering was never observed in cultures growing in 10 fc intensity or in darkness. In addition, flowering occurred only in cultures in which sugar was included in the medium. All explants which flowered had some attached cotyledonary tissue; however, those bearing more than one half of a cotyledon did not flower under any of the light intensities. The data for the development of the different sized explants under the different light intensities are summarized in table I.

Table I

The Development of Sunflower Explants Growing in Aseptic Culture on a Medium Containing 2% Sucrose *

Type of explant	Number of explants in each sub-series			
	800 fc	400 fc	10 fc	Dark
Plumule only				
Flowers	0	0	0	0
No flowers	48 **	42 **	16	32
Plumule and $\frac{1}{8}$ cotyledon				
Flowers	17	6	0	0
No flowers	10	18	30	15
Plumule and $\frac{1}{4}$ cotyledon				
Flowers	7	1	0	0
No flowers	16	21	26	16
Plumule and $\frac{1}{3}$ cotyledon				
Flowers	4	2	0	0
No flowers	32	16	16	17
Plumule and $\frac{1}{2}$ cotyledon				
Flowers	1	0	0	0
No flowers	32	30	25	17
Plumule and 1 cotyledon				
Flowers	0	0	0	0
No flowers	16	18	16	15

* Flowering did not occur in explants cultured on the same kind of medium except for omission of sucrose. Photoperiod 16 hrs and temperature 26° C.

** No growth.

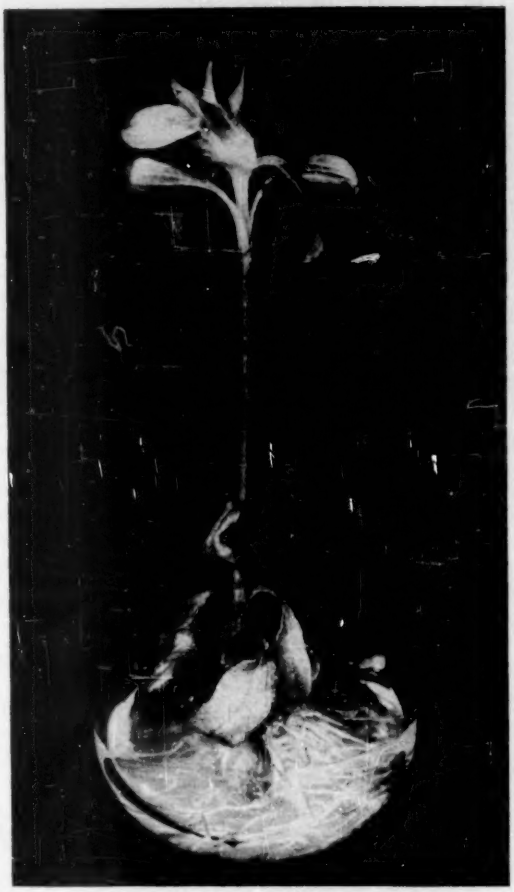

Fig. 1. Flowering sunflower explant, approximately × 1.4, plumule plus $\frac{1}{3}$ cotyledon, 85 days, three ray flowers open.

The explants which flowered were similar in appearance and followed the same general pattern of development. At anthesis these explants ranged from 2 to 6 cm in height. They had 3 to 7 pairs of small, green leaves, an inflorescence consisting of a few ray flowers, and a root system which was relatively small. The explant shown in figure 1 is representative of this group.

The development of this explant was typical of the flowering cultures. Roots were visible on the 56th day when the first internode measured 0.1 cm and the second internode 0.3 cm. The photograph was made on the 85th day when three ray flowers had expanded.

Flower buds never formed on explants in which the root system developed early. In many of the non-flowering cultures, roots were visible as early as 4 days after transfer. Root systems which developed early under 800 or 400 fc illumination grew rapidly

to relatively great size. During this period of root growth elongation of the stem slowed, ceased and the stem subsequently died. The roots of flowering explants appeared no earlier than 28 days after excision, and the root system always remained small. Visible roots were not a prerequisite to flowering since one explant blossomed in their absence.

All flowering explants were characterized by slow stem elongation and short internodes, at least up to the time of flower bud initiation. Thereupon considerable elongation of the internodes occurred in some explants. During the growth of the stem of flowering explants several pairs of leaves always developed prior to formation of the terminal flower bud.

A number of explants died before anthesis, probably from desiccation, since the medium dried appreciably during the long growth period.

Discussion

The mechanism which mediates the flowering reaction in these sunflower explants is not readily apparent. No floral initiation occurred in either dark or low light intensity, consequently it seems that a minimum light intensity is necessary. Since the flowering of certain plants growing in complete darkness has been reported (3), the mechanisms are apparently quite different.

Although the production of a "minimal leaf number" preceding flower initiation apparently is true for some species (Purvis (6) and others), there is no substantiation for it in this work. The number of nodes in flowering explants varied from 3 to 7, but more than 3 nodes often developed in cultures in 10 fc of light or in the dark without flower formation. Also, in certain explants growing on a sugarless medium under 800 fc of light 14 nodes were present without any indication of flower bud initiation.

Although flowering in these experiments might have been mediated through the production of a flowering hormone or by the establishment of certain nutritional levels, there are other possible explanations. Among them is the possibility that light-inactivated flower-inhibiting materials are present in the embryo and are stored principally in the cotyledons. Thus flowering might have been repressed until the inhibitor was reduced below a critical concentration in the plumule. This supposition is given some weight by the fact that flowering occurred with greatest regularity in explants with the smallest amount of attached cotyledonary tissue and failed to occur in the absence of light. It was pointed out earlier that flowering did not occur in explants bearing more than one half a cotyledon even under the highest light intensity. In general the proportionate number of flowering explants increased when the plumules bore progressively smaller pieces of cotyledon and when they were exposed to the highest light intensity.

It is impossible to state whether the presence of cotyledonary tissue is, or is not, a requisite to flowering because the explants consisting of plumules without attached cotyledonary tissue did not grow in 400 or 800 fc of light nor did they flower under 10 fc of light or in the dark.

Although flowering was confined to certain of the experimental conditions used, it is probable that there are other experimental conditions which are also favorable to floral initiation.

Summary

Plumules bearing different amounts of cotyledonary tissue were excised from germinating sunflower seeds and were cultured aseptically on a synthetic medium. Various series of explants grew in different intensities of fluorescent light and in the dark. Flowering occurred with greatest regularity in explants bearing small pieces of cotyledon and in the highest light intensity used. No flowering occurred if the light intensity was less than 400 fc, if sucrose was omitted from the medium, or if there was more than one-half cotyledon present. Flowers never developed in cultures in which the root system formed early. The formation of root systems of flowering cultures was always late and they remained relatively small. The existence of a light sensitive flower-inhibiting material is postulated.

The writer is indebted to Dr. B. S. Meyer for his helpful suggestions and guidance throughout the course of this study.

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STUDIES ON PLANT METABOLISM. IV. COMPARATIVE EFFECTS OF 2,4-DICHLOROPHENOXYACETIC ACID AND OTHER PLANT GROWTH REGULATORS ON PHOSPHORUS METABOLISM IN BEAN PLANTS (CONTINUED)

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EDITOR'S NOTE: Due to an error in correcting galley proof the two tables published below were omitted from the article of Fang and Butts in Plant Physiology, volume 29, pp. 365–368, July, 1954. These tables should be considered an integral part of this paper by Fang and Butts. Signed, THE EDITOR.

TABLE II

EFFECTS OF 2,4-D AND INDOLEACETIC ACID ON INCORPORATION AND DISTRIBUTION OF P^{32} IN THE WATER SOLUBLE FRACTION OF BEAN PLANTS (EACH GROUP CONSISTED OF 8 PLANTS)

	Leaf			Stem		
	Control	2,4-D treated	IAA treated	Control	2,4-D treated	IAA treated
		50 μgm per plant	*250 μgm per plant*		*50 μgm per plant*	*250 μgm per plant*
P^{32} activity per 10 μgm total P in H_2O extract	2,240 cpm	488 cpm	1,070 cpm	5,060 cpm	2,860 cpm	3,110 cpm
Total H_2O soluble P^{32} activity in plant part	4.6×10^5 cpm	1.5×10^5 cpm	3.1×10^5 cpm	5.8×10^5 cpm	9.5×10^5 cpm	5.8×10^5 cpm
Chemical Analysis *						
Inorg. P in H_2O extract	65.1 %	68.4 %	66.8 %	78.9 %	62.7 %	74.5 %
Results from P^{32} chromatograms **						
Inorg. P^{32} in H_2O extract	77.4 %	80.9 %	78.2 %	69.1 %	57.5 %	69.9 %
P^{32} incorporated in						
Glucose-1-phosphate	11.2 %	6.4 %	9.2 %	15.3 %	18.4 %	16.0 %
Hexosediphosphate	6.5 %	4.3 %	7.1 %	3.3 %	16.6 % †	5.4 %
Phosphoglyceric acid	1.7 %	2.5 %	1.6 %	3.7 %	3.5 %	3.0 %

* Average of duplicate determinations.
** Values were obtained from the average of 3 chromatograms.
† The P^{32} activity was greatly increased at that region, but it is not entirely hexosediphosphate.

TABLE III

EFFECTS OF INDOLEBUTYRIC ACID AND α-NAPHTHALENEACETIC ACID ON INCORPORATION AND DISTRIBUTION OF P^{32} IN THE WATER SOLUBLE FRACTION OF BEAN PLANTS (EACH GROUP CONSISTED OF 8 PLANTS)

	Leaf			Stem		
	Control	IBA treated	NAA treated	Control	IBA treated	NAA treated
		250 μgm per plant	*250 μgm per plant*		*250 μgm per plant*	*250 μgm per plant*
P^{32} activity per 10 μgm total P in H_2O extract	1,760 cpm	1,680 cpm	1,288 cpm	2,490 cpm	2,535 cpm	2,320 cpm
Total H_2O soluble P^{32} activity in plant part	7.34×10^5 cpm	3.70×10^5 cpm	4.17×10^5 cpm	4.15×10^5 cpm	2.98×10^5 cpm	4.29×10^5 cpm
Chemical Analysis *						
Inorg. P in H_2O extract	66.2 %	76.5 %	72.5 %	75.2 %	72.8 %	65.8 %
Results from P^{32} chromatogram **						
Inorg. P^{32} in H_2O extract	69.9 %	81.5 %	80.4 %	66.4 %	70.9 %	64.5 %
P^{32} incorporated in						
Glucose-1-phosphate	11.3 %	7.0 %	7.0 %	11.1 %	10.2 %	11.1 %
Hexosediphosphate	4.3 %	3.6 %	4.5 %	8.3 %	7.4 %	9.4 %
Phosphoglyceric acid	2.2 %	1.9 %	1.2 %	1.9 %	2.5 %	1.9 %

* Average of duplicate determinations.
** Values were obtained from the average of 3 chromatograms.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Election:* The American Society of Plant Physiologists elected Alden S. Crafts, Department of Botany, University of California, Davis, California, president; Harry A. Borthwick, U. S. Department of Agriculture, Bureau of Plant Industry, Plant Industry Station, Beltsville, Maryland, vice-president; Paul J. Kramer, Department of Botany, Duke University, Durham, North Carolina, executive committeeman; T. C. Broyer, Department of Plant Nutrition, University of California, Berkeley, California and Arthur W. Galston, Division of Biology, California Institute of Technology, Pasadena, California, members of the Editorial Board of *Plant Physiology*. All officers will assume their new duties January 1, 1955.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Annual Meeting, Gainesville, Florida, September 1954:* The American Society of Plant Physiologists held its 29th annual meeting September 5th to 8th, 1954 on the campus of the University of Florida, Gainesville, Florida. Our Society again cooperated with the Physiological Section of the Botanical Society of America in arranging the program. Four invited papers were presented at an intersociety symposium on "Experimental and Design Topics." In the ten regular sessions 130 contributed papers were presented; of this number, 23 were received through the Botanical Society—about half of the 23 being from members of our Society.[1]

At the annual banquet, the 30th anniversary of the founding of the Society was marked by an address given by Dr. Charles A. Shull, the first president of our Society; a short address by T. C. Broyer, our retiring president; announcement of the Stephen Hales Award, and the announcement of a Charles Reid Barnes Life Membership Award.

Executive Committee Meetings, September 5th and 6th: Reports of the president, secretary-treasurer, secretary and editor were received and referred to the secretary's file.

Dr. Stanfield expects to be able to publish a new directory for the Society by the early spring of 1955.

It was suggested to the secretary that printing of the abstracts be continued. The secretary is also instructed to explore the possibility of having the abstracts published as bulletins or supplements suitable for binding with volumes of *Plant Physiology*.

Dr. Goddard reported that since becoming editor in 1953 he has handled 244 papers. Eighty-seven of these were on hand at the time of assuming office. Sixty-five papers have been declined or withdrawn. Most of the backlog had been cleared. Publication within about 6 months after receipt is the goal. This goal will be partially achieved before the end of the year. At present, it is anticipated the last issue of the current volume of *Plant Physiology* will appear in late December or early January.

A budget of $18,500.00 was voted to Dr. Goddard for editorial expenses, cost of publication and distribution of *Plant Physiology*.

Reports of several committees were heard. Two committees were disbanded and no new ones formed. Dr. van Overbeek's committee on "Nomenclature of Growth Substances" was dissolved. Its report has already been published. Dr. Hamner's committee on "Artificial Light for Growth of Plants" was dissolved at Dr. Hamner's request. The committee on "Professional Status and Training" is to be continued though Dr. Bonner has resigned as chairman.

Several new appointments were made. Dr. Sterling Hendricks and Dr. Orlin Biddulph were appointed to the Editorial Board. Dr. J. P. Bennett was made our second representative on the American Association for the Advancement of Science Council. Our new representative on the National Research Council is Dr. F. C. Steward. Dr. Sterling Hendricks was reappointed to the finance committee. Our program committee for the annual meeting to be held in September 1955 at Michigan State is to be composed of Aubrey W. Naylor, Carroll Swanson and Sylvan Wittwer.

A decision was quickly reached to meet with the American Institute of Biological Sciences at Stanford University in 1957.

Considerable time was spent on new business. A change in membership dues from $6.00 to $8.00 for all members was voted. The change was made contingent upon approval by the general membership present at the annual business meeting to be held September 7th. Minor changes in the By-Laws, all involving changes in time of performing certain duties, were made. These changes were made necessary by the fact we now meet in September instead of December. J. S. Canner Company was given permission to "Micro-Card" volumes 1 to 5 and parts 1 and 2 of volumes 20 and 21 of *Plant Physiology*.

Encouragement was given by the executive committee for the formation of a Midwestern Section of the Society to take the place of several local chapters. Those persons interested in supporting and actively aiding in organizing the Midwestern Section should get in touch with Dr. J. B. Hanson at the University of Illinois, Urbana, Illinois. It is anticipated that an organizational meeting including contributed papers will be held in the spring of 1955.

The executive committee went on record favoring cooperation with the International Association of Plant Physiologists organized during the International Botanical Congress meetings held in Paris in July of this year. Through such cooperation international exchange of ideas and of persons should be increased and physiology should be better represented in the International Botanical Congress. Furthermore, physiology now becomes eligible for support from UNESCO or other international bodies for the holding of symposia on special topics.

Approval was given of the action taken by the executive committee and the executive council of the

[1] About 75 copies of the Gainesville program are still available at 50¢ each. They may be obtained from the Secretary, Duke University, Durham, North Carolina.

American Institute of Biological Sciences (A.I.B.S.) to change its constitution. The changes will permit the A.I.B.S. to act as a free agent independent of the National Research Council, if it so wishes, and also provides for additional types of membership.

Annual Business Meeting, September 7th: Following a reading of the minutes of the 1953 meeting and a summary of actions taken by the Executive Committee, the meeting was thrown open for discussion. Approximately 60 members were in attendance.

Extensive debate was aroused by introduction of the question of raising dues to members. Dr. Stanfield presented convincing evidence that services now given by the Society considerably exceed the dues being paid by the members. An amendment to the proposal to raise dues, which would permit special memberships other than those now recognized, lost by a narrow margin. By unanimous vote, dues for all members—domestic and foreign—were raised to $8.00.

Dr. Arthur Galston introduced a resolution that the American Society of Plant Physiologists go on record as opposing the apparent infiltration of influence of political feelings into the making of grants in support of scientific research. A lively debate followed—particularly over the wording of the resolution. After slight modification, the resolution was finally accepted by majority vote and the secretary instructed to transmit the resolution to the appropriate authorities. The resolution reads as follows:

"It has come to the attention of the American Society of Plant Physiologists that political criteria are now being employed by the Department of Health, Education and Welfare in awarding of research grants through the U. S. Public Health Service. We deplore this practice, for we feel the introduction of such political considerations into scientific effort to be contrary to American tradition and to be harmful to science as a whole. We respectfully urge a return to former selection procedures, in which only the scientific merit of the research proposal and the integrity and research record of the investigator are judged."

No further business was considered.—Respectfully submitted, AUBREY W. NAYLOR, Secretary.

THE STEPHEN HALES AWARD: Professor Folke Skoog of the University of Wisconsin was granted the Stephen Hales Award at the annual meeting of the American Society of Plant Physiologists, Gainesville, Florida. In the committee's citation, Dr. Skoog was described as an outstanding worker whose researches in the fields of auxin physiology, culture of fresh water algae and tissue culture of higher plants have contributed largely to the science of plant physiology.

Dr. Skoog was born in Sweden in 1908. He graduated from the California Institute of Technology in 1932 with a doctorate from that institution in 1936. He was a National Research Council fellow at the University of California the following year, instructor and research associate at Harvard from 1937 to 1941, associate professor at The Johns Hopkins University from 1941 to 1944, and associate professor and professor at Wisconsin since 1947.

CHARLES REID BARNES LIFE MEMBERSHIP AWARD: Dr. Andrew Edward Murneek, of the University of Missouri, was named recipient of the 29th Charles Reid Barnes Life Membership Award, at the annual banquet of the American Society of Plant Physiologists, Gainesville, Florida, on September 6th, 1954.

Dr. Murneek was born at Talsen, Latvia, May 21, 1888. He obtained his B.S. from Oregon State College in 1917 and was a fellow at Iowa State College in 1917–1918; receiving his M.S. in 1918. Dr. Murneek then moved to Wisconsin where he obtained his Ph.D. in 1925. Before this he served as Assistant Research Horticulturist at Oregon State College from 1918 to 1923. On completion of his doctorate, he became associated with the University of Missouri where he has remained until the present time.

Dr Murneek was vice-president of the American Society of Plant Physiologists in 1927; he served as secretary-treasurer from 1933 to 1935 and was elected president in 1935.

Dr. Murneek is well known for his work on the physiology of reproduction of plants; photoperiodism; fruit pollination and sterility; nutrition; biennial fruiting and plant hormones. He was also one of the initial contributors to volume 1, number 1 of *Plant Physiology*.

MEETING OF PLANT CHEMISTS AND BIOCHEMISTS: An organizational and paper reading session for plant chemists and biochemists was held at Columbia University on October 23rd. The new group, comprising about 50 persons in the lower New England and Middle Atlantic area, voted to hold annual meetings and to survey the possibilities for coordinating information and publications in the broad field of plant chemistry. Prof. R. F. Dawson of Columbia was elected recording secretary, and professors D. R. Sprinson of Columbia and R. A. Barnes of Rutgers were appointed to serve on the program committee for 1955. During the scientific session papers were read on the chemistry and biosynthesis of natural products by Drs. Ulrich Weiss, Marjorie Anchel, Franz Moewus and R. A. Barnes. Pathways of carbohydrate dissimilation and of nitrate reduction were discussed by Drs. Martin Gibbs and Alvin Nason. Chairmen of the session were Drs. H. B. Vickery of New Haven and R. C. Anderson of Brookhaven National Laboratory.—R. F. DAWSON, Secretary.

ANNUAL REVIEW OF PLANT PHYSIOLOGY, VOLUME IV.—D. I. Arnon, Editor. Annual Reviews, Inc., Stanford, California. 453 pages. 1953. $7.00.

The fourth volume of this series includes the following fifteen articles: Nitrogen Metabolism of Higher Plants by J. G. Wood; Photosynthesis by A. H. Brown and A. W. Frenkel; The Use of Respiratory Inhibitors by W. O. James; Organic Acids in Plant Metabolism by R. H. Burris; Hematin Compounds in Plants by R. Hill and E. F. Hartree; The Relation of Chemical Structure to Biological Activity in Growth Substances by H. Veldstra; The Physiology of Fruit

Growth by J. P. Nitsch; The Physiology of Root Growth by H. Burström; Herbicides by A. S. Crafts; Fluorescent Substances in Plants by R. H. Goodwin; The Biogenesis of Terpenes by A. J. Haagen-Smit; Physiology of Mycorrhizal Relations in Plants by E. Melin; The Effect of Temperature on Plant Growth by F. W. Went; Physical Properties of Protoplasm by H. I. Virgin; The Plant Sterols by W. Bergmann.

Even a cursory perusal of the contents of this volume will make it manifestly clear that the variety and treatment of subject matters should render it of distinct value to the specialized biochemist and horticulturist alike. Papers on herbicides, temperature effects, nitrogen metabolism and fruit growth will appeal especially to agronomists, pomologists, floriculturists, foresters, and other horticulturists who are interested in current advances in their related areas. This alignment of topics may lead one to visualize a genuine appeal of subsequent volumes to a wider audience of plant scientists.

Exemplifying the kind of appraisal that will assuredly not be restricted to a highly specialized audience is F. W. Went's eminently good review of temperature effects on growth responses within the temperature range of usually normal physiological activity. Injury effects at temperature extremes are pointedly not considered in his paper. Thermoperiodicity, both in terms of vegetative and reproductive response, is emphasized and Went's detailed studies of diurnal periodicity are clearly summarized. Included also are brief treatments of temperature on dormancy, germination, vernalization, color intensity in flowers, and some miscellaneous effects. The author is critical of the adequacy of approaches to analyses of field temperature data on growth, including "heat sums," "degree days," and "crop logs," with some argument for his stand.

A. S. Crafts' very readable treatment of herbicides is the third analysis on this subject in the series and reinforces the editorial policy of dealing primarily with areas of intense research activity and published output rather than complete coverage of many phases of plant physiology. Included are treatments of absorption mechanisms, translocation, mechanisms of auxin action, fate of 2,4-dichlorophenoxyacetic acid in plants, antiauxins, variety of plant responses, crop injury and modes of action of several other categories of herbicides including carbamates, phenylmercuric acetate, potassium cyanate, Endothal, 3-(*p*-chlorophenyl)-1, 1-dimethyl urea, maleic hydrazide, N-1-naphthylphthalamic acid, and others.

Brown and Frenkel's handling of photosynthesis appraises more recent findings dealing with quantum requirements, biochemical pathways of carbon, photosynthetic enzymes, and phosphorus metabolism. Of crucial significance to all biologists is the brief section which weighs evidence purporting to support the rather widely accepted theory of water as the sole source of photosynthetic oxygen. Although some textbooks are stating with finality that water provides all photosynthetic oxygen, the authors point to recent tracer oxygen data which advance the claim that carbon dioxide may be a source of something of the order of a fifth to as much as a third of such oxygen. Brown and Frenkel conclude that the evidence available to them is simply inadequate to support or deny the intrenched theory of water as the sole oxygen precursor.

The fact that a glossy paper was used to reproduce some molecular models points up the added fact that a somewhat higher quality paper throughout might be preferable. This criticism may be in the nature of a peccadillo, however, since the paper is of an acceptable quality.

This is a scholarly contribution and represents the efforts of a distinguished editorial staff and reviewers of considerable stature who have been judiciously selected for their broad knowledge of current research. Their combined energies will serve the useful purpose of keeping plant scientists aware of significant developments in a discipline which is characterized by increasing dichotomy and complexity. Easy accessibility may be had through these volumes to a summary of developments outside the very specific problem with which an individual researcher must be primarily concerned.

Volume 4 is enhanced by a total of almost three thousand literature citations and very complete author and subject indices.—THEODORE T. KOZLOWSKI, *University of Massachusetts, Amherst, Massachusetts.*

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

Organized in 1924 for the advancement of Plant Physiology as a pure and applied field of botanical science

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OFFICERS FOR 1953–54

President: Mr. Theodore C. Broyer,
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Secretary: Dr. Aubrey W. Naylor,
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Any one actively interested in Plant Physiology is eligible for membership in the Society. Candidates for membership must be recommended by two members, and approved by executive committee, or by the vote of the Society at some annual or special meeting.

The payment of the annual dues to the Society entitles the members to receive the official journal, Plant Physiology. They will receive in addition the regular bulletins and announcements sent out periodically by the secretary. Members are invited to take a personal interest in the growth and development of the Society.

Manuscripts for Plant Physiology should be addressed to Prof. David R. Goddard, Botanical Laboratories, University of Pennsylvania, Philadelphia, Pennsylvania. Drawings and photographs should not exceed 8.5 × 11 inches in size, should be numbered correctly on the back, and separate typewritten legends included with the text of the manuscript. Tables and cuts should be used sparingly, and the text presented in concise form. Instructions to authors for preparation of manuscripts are given in the January number, Volume 29, 1954, of Plant Physiology, 111–112.

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